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PATENT

PATENT APPLICATION

TO ALL WHOM IT MAY CONCERN:

Be it known that we, ROBERT OLMSTED, PAULA KEITH, SERGEY DRYGA, IAN CALEY, MAUREEN MAUGHAN, ROBERT JOHNSTON, NANCY DAVIS and RONALD SWANSTROM, have invented new and useful improvements in

ALPHAVIRUS VECTORS AND VIROSOMES
WITH MODIFIED HIV GENES FOR USE IN VACCINES

for which the following is a specification.

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ALPHAVIRUS VECTORS AND VIROSOMES WITH MODIFIED HIV GENES FOR USE IN VACCINES

This application is a continuation-in-part of and claims priority to, U.S. application Serial No. 09/902,537, filed July 9, 2001 (abandoned), which claims priority to provisional application Serial No. 60/216,995, filed July 7, 2000, which applications are incorporated by reference herein in their entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to vaccines using viral antigens, and in particular, to vaccines for the treatment and prevention of human immunodeficiency virus (HIV) infection. The vaccines of this invention comprise alphavirus RNA replicon systems which contain nucleic acid sequence encoding antigens for eliciting an immune response to HIV.

Background

The successful control of the AIDS epidemic will require an effective vaccine for human immunodeficiency virus type 1 (HIV) that significantly reduces or prevents the spread of infection. Currently, several viral vector systems as well as naked DNA are at various stages of pre-clinical and clinical evaluation as candidate HIV vaccines. Recombinant poxviruses are the most widely studied virus vectors and are furthest along in clinical development (e.g., ALVAC).

The alphavirus-based replicon particle systems, such as the ones described in U.S. Patent No. 5,792,462 and herein referred to as "VRPs," have multiple distinct properties that make them attractive as an HIV vaccine delivery technology. These properties include: natural targeting to and expression in lymphoid tissues (an optimal site for induction of an immune response); high antigen expression levels, e.g., up to 20% of total cell protein; induction of balanced humoral, cellular, and mucosal immune

responses; sustained efficacy over multiple simultaneous or sequential inoculations of the vector; and a high margin of safety.

Venezuelan equine encephalitis virus (VEE) is a member of the Alphaviruses group, which also includes the prototype Sindbis virus (SIN) and Semliki Forest virus (SFV), and is comprised of enveloped viruses containing plus-stranded RNA genomes within icosahedral capsids (Strauss, 1994). Alphavirus genomes are: approximately 11.5 kb long, capped, polyadenylated, and infectious under appropriate transfection conditions. The nucleocapsid is composed of 240 molecules of the capsid protein arranged as a T=4 icosahedron, and is surrounded by a lipoprotein envelope (Paredes *et al.*, 1993). Protruding from the virion surface are 80 glycoprotein spikes, each of which is a trimer of virally encoded E1 and E2 glycoprotein heterodimers. The virions contain no host proteins.

Alphaviruses share replication strategies and genomic organization. The complete replicative cycle of alphaviruses occurs in the cytoplasm of infected cells. Expression from the alphavirus genome is segregated into two regions. The four enzymatic nonstructural proteins (nsP1-nsP4) are synthesized from the 5' two-thirds of the genome-length RNA and are required for RNA replication. Immediately following infection, the nsPs are produced by translation of parental genomes and catalyze the synthesis of a full-length negative-sense copy of the genome. This serves as a template for the synthesis of progeny plus-stranded genomes.

The negative-sense copy of the genome also serves as the template for the synthesis of subgenomic mRNA at approximately 10-fold molar excess relative to genomic RNA in infected cells (Schlesinger and Schlesinger, 1990). Synthesis of subgenomic 26S mRNA is initiated from the highly active internal 26S mRNA promoter, which is functional only on the negative-sense RNA. The subgenomic mRNA corresponds to the 3' one-third of the genome and encodes the alphavirus structural proteins.

Full-length, infectious cDNA clones of the RNA genome of VEE (Davis *et al.*, 1989) have been constructed, a panel of mutations which strongly attenuate the virus have been identified (Johnston and Smith, 1988; Davis *et al.*, 1990), and various constellations of these attenuating mutations have been inserted into the clones to generate several live attenuated VEE vaccine candidates (Davis *et al.*, 1991; 1995b; Grieder *et al.*, 1995). The resulting vaccine candidates are avirulent and provide complete protection against lethal virus challenge in rodents, horses and nonhuman primates.

The alphavirus VRPs are propagation defective, single cycle vectors that contain a self-amplifying alphavirus RNA (replicon RNA) in which the structural protein genes of the virus are replaced by a heterologous antigen gene to be expressed. Alphavirus VRPs are typically made in cultured cells, referred to as packaging cells. Following introduction into mammalian cells, the replicon RNA is packaged into VRP by supplying the structural proteins in "trans," i.e., the cells are co-transfected with both replicon RNA and one or more separate helper RNAs which together encode the full complement of alphavirus structural proteins. Importantly, only the replicon RNA is packaged into VRP, as the helper RNA(s) lack the *cis*-acting packaging sequence required for encapsidation. Thus, the VRPs are defective, in that they can only infect target cells in culture or *in vivo*, where they express the heterologous antigen gene to high level, but they lack critical portions of the VEE genome (i.e., the VEE structural protein genes) necessary to produce virus particles which could spread to other cells.

Delivery of the replicon RNA into target cells (for vaccination) is facilitated by the VRP following infection of the target cells. In the cytoplasm of the target cell, the replicon RNA is first translated to produce the viral replicase proteins necessary to initiate self-amplification and expression. The heterologous antigen gene is encoded by a subgenomic mRNA, abundantly transcribed from the replicon RNA, leading to high level expression of the heterologous antigen gene product. Since the VEE structural protein genes are not encoded by the replicon RNA delivered to the target cell, progeny virion particles are not assembled, thus limiting the replication to a single cycle within

the infected target cell. Experimental VRP vaccines have been successful in vaccinating rodents against influenza virus, Lassa fever virus and Marburg virus (Pushko *et al.*, 1997; Hevey *et al.*, 1998). In nonhuman primates, VRP vaccines have demonstrated complete efficacy against lethal Marburg virus challenge (Hevey *et al.*, 1998), shown partial but significant protection against SIV infection and disease (Davis *et al.*, 2000) and induced an anti-HA response at a level consistent with protection of humans against influenza virus infection.

The alphavirus based replicon vector systems, and in particular the VEE-based systems, present several advantages in vaccination, including safety and high immunogenicity/efficacy. VEE is unique among the alphaviruses in that a live attenuated IND VEE vaccine, TC-83, (Kinney *et al.*, 1989; Kinney *et al.*, 1993) has been inoculated into approximately 8,000 humans. This allows direct safety and efficacy comparisons between human, nonhuman primate and rodent responses to the same VEE derivative. A large body of experience strongly suggests that the animal models generally reflect the human susceptibility and disease course, except that mice are far more susceptible to lethal VEE disease than humans or nonhuman primates. Furthermore, the VEE replicon vectors express high levels of the gene of interest in cell culture, and *in vivo* expression is targeted to lymphoid tissues, reflecting the natural tropism mediated by the VEE glycoproteins. Cells in the draining lymph node of VRP-inoculated mice contain detectable amounts of the desired gene product within hours of inoculation. This expression continues for up to five days.

To date, VRP vector vaccines have been used in over 2000 rodents and in 94 macaques at doses up to 5×10^8 i.u., with no indication of any clinical manifestations.

In work reported by Pushko *et al.* (1997), individual mice were immunized sequentially with Lassa virus N-VRP and influenza virus HA-VRP. Groups of mice, which received two inoculations of 3×10^4 or 3×10^6 i.u. of Lassa N-VRP followed by two inoculations of 2×10^5 i.u. of HA-VRP, all responded with serum antibodies to both antigens. The level of anti-influenza antibody induced in these sequentially

inoculated mice was equivalent to a control group, which received two inoculations of buffer followed by two inoculations of 2 x 10^5 i.u. of HA-VRP. All HA-VRP immunized mice were completely protected against influenza virus challenge. Furthermore, sequential immunization of mice with two inoculations of N-VRP prior to two inoculations of HA-VRP induced an immune response to both HA and N equivalent to immunization with either VRP construct alone. Primary and booster immunization with a VRP preparation expressing an immunogen from one pathogen did not interfere with the development of a protective response to subsequent primary immunization and boosting with VRP expressing an immunogen from a second pathogen, thus showing that the VRP-based system can be used to induce immunity to a variety of pathogens in the same individual over time.

Four macaques were inoculated subcutaneously at week 0 with 10^5 i.u. each of SIV-gp160-VRP (*env*) and SIV MA/CA-VRP (*gag*), boosted by the same route at week 7 with 10^7 i.u. of each VRP vaccine, and intravenously at weeks 12 and 20 with 5 x 10^8 i.u. of each VRP. Two control animals were inoculated with equivalent doses of HA-VRP (haemagglutinin, a glycoprotein from influenza virus), and two with the vehicle only. The four SIV-VRPs immunized monkeys received subcutaneously an additional dose of 2 x 10^7 i.u. of gp140-VRP at week 41, followed by a final boost of 2 x 10^7 i.u. each of gp140-VRP and MA/CA-VRP at week 49. Four weeks after the final immunization, all eight macaques were challenged intravenously with the pathogenic virus, SIVsmE660.

After these inoculations, three of four test macaques had measurable CTL-specific killing directed against both SIV *gag* and *env*, all four had gp160 IgG antibody by ELISA, and the three animals which harbored SIV-specific CTL also showed neutralizing antibody to SIVsmH-4.

Four of four vaccinated animals were protected against disease for at least 16 months following intravenous challenge with the pathogenic SIV swarm, while the two vehicle controls required euthanasia at week 10 and week 11, post challenge. In two of

the vaccinees, plasma virus levels were below the limit of detection by branched chain DNA assay. At 64 weeks post challenge, all four vaccinated animals showed no clinical signs of disease. One animal remained vDNA negative at 64 weeks.

The results of this highly pathogenic challenge demonstrated that the immune response induced by vaccination with SIV-VRP was effective in preventing early mortality and increasing the ability to suppress challenge virus replication. The ability to control SIV replication and reduce viral load to undetectable levels was closely correlated with the strongest measurable antibody and cellular immune responses.

While these results are encouraging, the level of protection obtained would not be acceptable for a human vaccine against HIV infection. Thus, there remains a need for a robust, effective and safe vaccine against HIV infection in humans. Development of a HIV vaccine comprising the complete, or immunogenic fragments of the, *gag* gene (Gag-VRP), an immunogenic portion of the *pol* gene (Pol-VRP), and the complete, or immunogenic fragments of the, *env* gene (Env-VRP), would increase the diversity of available CTL epitopes substantially and thus address this need.

SUMMARY OF THE INVENTION

The present invention provides a composition comprising two or more isolated nucleic acids selected from the group consisting of an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles by the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity.

Also provided is a composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles by the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to remove protease, integrase and RNase H regions and to inhibit reverse transcriptase activity, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle.

In addition, the present invention provides a composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle, and further wherein the alphavirus replicon particles comprise a replicon RNA or at least one structural protein which comprises one or more attenuating mutations.

10. A method of making a population of alphavirus replicon particles of this

invention is provided herein, comprising:

A) **(a)** providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

B) **(a)** providing a second helper cell for producing a second population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a

gag gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

C) **(a)** providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic

fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles.

Also provided is a method of making a population of alphavirus replicon particles, comprising:

A) **(a)** providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA

comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

B) **(a)** providing a second helper cell for producing a second population of infectious, replication defective alphavirus particle, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic

fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic

fragment thereof is modified to inhibit reverse transcriptase activity, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles.

Furthermore, the present invention provides a composition comprising two or more isolated nucleic acids selected from the group consisting of an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human

immunodeficiency virus, an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof.

In addition, the present invention provides a composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle.

Also provided herein is a composition comprising a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a

human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon particle, and further wherein the alphavirus replicon particles comprise a replicon RNA or at least one structural protein which comprises one or more attenuating mutations.

In these embodiments, the *gag* gene product or immunogenic fragment thereof can be modified by mutation of the second codon, whereby a glycine is changed to an alanine and the *pol* gene product or immunogenic fragment thereof can be modified by mutation of the nucleotide sequence encoding the active site motif, whereby YMDD is changed to YMAA or HMAA. In addition, the *pol* gene product or immunogenic fragment thereof is modified to remove protease, integrase and RNase H regions and to produce only p51 of the *pol* gene product or immunogenic fragment thereof.

The present invention provides a method of making a population of alphavirus replicon particles, comprising:

A) **(a)** providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and

furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

B) **(a)** providing a second helper cell for producing a second population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said

replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles.

An additional method of making a population of alphavirus replicon particles is provided, comprising:

A) **(a)** providing a first helper cell for producing a first population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

B) **(a)** providing a second helper cell for producing a second population of infectious, replication defective alphavirus particle, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

C) (a) providing a third helper cell for producing a third population of infectious, replication defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein;

and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the population of alphavirus replicon particles.

In each of the methods above, the alphavirus replicon RNA of at least one of the first helper cell, the second helper cell and the third helper cell can comprise sequence encoding at least one alphavirus structural protein and the first helper RNA and the one or more additional helper RNA(s) in the at least one of the first helper cell, the second helper cell and the third helper cell, can encode at least one other alphavirus structural protein not encoded by the replicon RNA.

Furthermore, in the methods above which recite attenuating mutations, only at least one of the first population of alphavirus particles, the second population of

alphavirus particles and the third population of alphavirus particles can comprise particles wherein at least one of the replicon RNA, the first helper RNA, and the one or more additional helper RNA(s) comprises one or more attenuating mutations.

The present invention further provides alphavirus particles produced by any of the methods of this invention.

The present invention further provides a method of inducing an immune response to human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the populations and/or compositions of this invention, in a pharmaceutically acceptable carrier.

Also provided herein is a method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the populations and/or compositions of this invention, in a pharmaceutically acceptable carrier.

Also provided by the present invention is an alphavirus replicon virosome comprising an alphavirus replicon RNA encapsidated by a lipid bilayer comprising alphavirus glycoproteins, E1 and E2, which in one embodiment, can be Venezuelan Equine Encephalitis glycoproteins E1 and E2.

A method of producing an alphavirus replicon virosome is further provided, comprising: a) combining alphavirus replicon RNA, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and b) gradually removing detergent, whereby alphavirus replicon virosomes are produced. Also provided is a virosome produced by this method.

Furthermore, the present invention provides a method of eliciting an immune response in a subject, comprising administering to the subject an immunogenic amount of the alphavirus replicon virosome of this invention in a pharmaceutically acceptable

carrier.

The present invention additionally provides a method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the alphavirus replicon virosome of this invention, wherein the virosome comprises alphavirus replicon RNA encoding one or more HIV immunogens.

In further embodiments, the present invention provides a composition comprising a population of alphavirus replicon virosomes comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon virosome.

Additionally provided herein is a composition comprising a population of alphavirus replicon virosomes comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the

gag gene product or the immunogenic fragment thereof and their release from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in inactivation of reverse transcriptase activity in the *pol* gene product or immunogenic fragment thereof, and wherein the nucleic acids are each contained within a separate alphavirus replicon virosome.

A method of producing a population of alphavirus replicon virosomes is provided herein, comprising:

A) (a) producing a first population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding an *env* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

B) (a) producing a second population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding a *gag* gene product or immunogenic fragment thereof, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

C) (a) producing a third population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding the *pol* gene product or immunogenic fragment thereof, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or

inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced; and

D) combining the first population of alphavirus replicon virosomes, the second population of alphavirus replicon virosomes and the third population of alphavirus replicon virosomes to produce the population of alphavirus replicon virosomes.

In addition, a method of producing a population of alphavirus replicon virosomes is provided, comprising:

A) (a) producing a first population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding and *env* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

B) (a) producing a second population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding and *gag* gene product or immunogenic fragment thereof, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced;

C) (a) producing a third population of alphavirus replicon virosomes by combining alphavirus replicon RNA comprising nucleic acid encoding the *pol* gene

product or immunogenic fragment thereof, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in inactivation of reverse transcriptase activity in the *pol* gene product or immunogenic fragment thereof, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and

b) gradually removing detergent, whereby alphavirus replicon virosomes are produced; and

D) combining the first population of alphavirus replicon virosomes, the second population of alphavirus replicon virosomes and the third population of alphavirus replicon virosomes to produce the population of alphavirus replicon virosomes of claim 48.

Furthermore, the present invention provides a method of inducing an immune response in a subject, comprising administering to the subject an immunogenic amount of the virosomes of this invention, in a pharmaceutically acceptable carrier.

Also provided is a method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an immunogenic amount of the virosomes of this invention, in a pharmaceutically acceptable carrier.

Additionally provided by this invention is a composition comprising heparin affinity-purified alphavirus replicon particles, wherein the alphavirus replicon particles comprise at least one structural protein which comprises one or more attenuating mutations, as well as a method of preparing heparin affinity-purified alphavirus particles, comprising:

a) producing alphavirus replicon particles, wherein the alphavirus replicon particles comprise at least one structural protein which comprises one or more attenuating mutations;

b) loading the alphavirus replicon particles of step (a) in a heparin affinity chromatography column;

c) eluting the particles from the column of step (b) with a salt gradient

(e.g., NaCl gradient); and

d) collecting the fraction from the column which contains the heparin affinity-purified alphavirus replicon particles.

In further embodiments, the present invention provides a method of producing VRP for use in a vaccine comprising:

a) producing a plasmid encoding the nucleotide sequence of an alphavirus replicon RNA;

b) producing a plasmid encoding the nucleotide sequence of one or more helper RNAs;

c) transcribing the plasmids of steps (a) and (b) into RNA *in vitro*;

d) electroporating the RNA of step (c) into a Vero cell line; and

e) purifying VRP from the Vero cell line of step (d) by heparin affinity chromatography. By this method, VRPs can be produced in large-scale.

In additional embodiments, the present invention provides an isolated nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof. This nucleic acid can be present in a composition and in a vector. Such a vector can be present in a cell. This nucleic acid can also be present in an alphavirus replicon particle.

The present invention further provides a method of making an alphavirus replicon particle comprising nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the

pol gene product or immunogenic fragment thereof, comprising

a) providing a helper cell for producing an infectious, defective alphavirus particle, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cell.

In the method described above, at least one of the replicon RNA, the first helper

RNA, and the one or more additional helper RNA(s) can comprise one or more attenuating mutations. The present invention additionally provides alphavirus replicon particle produced according to the above methods.

Further provided is a method of inducing an immune response in a subject, comprising administering to the subject an immunogenic amount of a composition comprising alphavirus replicon particles encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof in a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. DNA plasmid map of VEE replicon RNA encoding the HIV *gag* gene (p3-40.1.6). The plasmid is 12523 base pairs in length and encodes a single polyprotein encoding the four non-structural genes nsP1-4, the Clade C gag gene and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize prior to *in vitro* transcription is also noted.

Figure 2. DNA plasmid map of the capsid helper construct (p3-13.2.2). The plasmid is 5076 base pairs in length and encodes the VEE capsid gene (C) and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize DNA prior to *in vitro* transcription is also noted.

Figure 3. DNA plasmid map of the glycoprotein helper construct (p3-13.4.6). The plasmid is 6989 base pairs in length and encodes the VEE glycoprotein genes (E3,

E2, 6K and E1) and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize DNA prior to *in vitro* transcription is also noted.

Figure 4. DNA plasmid map of VEE replicon RNA encoding HIV *pol* (p51) gene (p13-60.2.14). The plasmid is 12379 base pairs in length and encodes a single polyprotein encoding the four non-structural genes, nsP1-4, the Clade C pol (p51) gene and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize prior to *in vitro* transcription is also noted.

Figure 5. DNA plasmid map of VEE replicon RNA encoding HIV *env* gene (pERK-Du151env). The plasmid is 13584 base pairs in length and encodes a single polyprotein encoding the four non-structural genes, nsP1-4, the Clade C env gene and antibiotic resistance marker, Kanamycin KN(R). The plasmid contains two promoter regions, the T7 polymerase promoter and the 26S RNA promoter. The unique NotI restriction enzyme site used to linearize prior to *in vitro* transcription is also noted.

Figure 6. Western immunoblot, demonstrating the expression of HIV proteins in baby hamster kidney (BHK) cells infected with VRPs. The outer lanes of the panel are standard molecular weight markers. Lane 1 is the expression from VRPs encoding the p51 (*pol*) gene. Lane 2 is the expression from VRPs encoding the gp160 (*env*) gene. Lane 3 is the expression from VRPs encoding the p55 (*gag*) gene. Arrows indicate proteins migrating with the apparent molecular weight of each respective protein.

Figure 7. Western immunoblot of cells infected with the Du151*env* VRP. At 18 hr post infection, the cells were lysed and the lysate run in a denaturing polyacrylamide gel. Proteins were transferred out of the gel onto a filter and the filter was probed with serum from subject Du151 using Western immunoblot methods. Lane

1, uninfected U87.CD4-CXCR4 cells. Lane 2, uninfected U87.CD4-CCR5 cells. Lane 3, infection of a mixed culture of U87.CD4-CXCR4 cells and BHK cells (mixtures were used as a positive control in case the U87 cells were refractory to infection by the VRP, which did not turn out to be the case). Lane 4, infected U87.CD4-CXCR4 cells. Lane 5, infected BHK cells. Lane 6, infection of a mixture of BHK cells and U87.CD4-CCR5 cells. Lane 7, infected U87.CD4-CCR5 cells. The positions of molecular weight of markers run in the same gel are shown on the right, and the inferred positions of gp160, gp120 and gp41 are shown on the left.

Figure 8. Micrographs of U87.CD4-CCR and BHK cells used to examine expression and syncytium formation of Du151 envelope expressed from the VEE replicon. U87.CD4-CCR5 cells alone (Panel 1), or a mixture of U87.CD4-CCR5 and BHK cells (Panel 2), BHK cells alone (Panel 3) and U87.CD4-CXCR4 cells (Panel 4) were infected with Du151 env VRP at a multiplicity of infection of 3 i.u. per cell. At 18 hours post infection, the cells were examined using light microscopy for the presence of syncytia. The U87.CD4-CCR5 in Panel 1 and 2 show clear syncytia, which was absent in the control cell types in the lower panels. In addition, no syntycia were seen in uninfected control cells or VRP-GFP infected cells (data not shown).

Figures 9A-C. Antigen-specific CTL response in mice to the HIV-1 Clade C VRP-Gag vaccine. Eight BALB/c mice were immunized twice, first at day 0 and again at day 28 with 10^3 i.u. (Panel A) or 10^5 i.u. (Panels B and C) VRP-Gag. Eight days (Panels A and B) or 49 days (Panel C) post-boost, spleen cells were isolated and stimulated *in vitro* with vaccinia virus expressing HIV Gag for 1 week. Chromium release assays were performed using vaccinia-Gag infected target cells (diamond symbols) or control vaccinia alone-infected sc11 target cells (square symbols). Clear HIV Gag-specific lysis was detected in animals vaccinated with the VRP-Gag vaccine.

Figure 10. Diagrammatic representation of the HIV-1 genome. Black bars indicate relative regions of the genome sequenced to generate phylogenetic sequence comparative data for Clade C *gag*, *pol* and *env* gene isolates.

Figure 11. Phylogenetic comparison of Du422 Clade C Gag isolate with referenced Clade C strains. Consensus clade A, B, D, Mal and SA strains are also shown. Du422 the vaccine strain had 95% amino acid sequence homology to the South African consensus Clade C sequence.

Figure 12. Phylogenetic comparison of Du151 Clade C isolate Env isolate with referenced Clade C strains. Du151 the vaccine strain had 93% amino acid sequence homology to the South African consensus Clade C sequence.

Figure 13. Phylogenetic comparison of Du151 Clade C isolate Pol isolate with referenced Clade C strains. Du151 the vaccine strain had 99% amino acid sequence homology to the South African consensus Clade C sequence.

Figure 14. Du422 HIV Gag expression as detected by immunofluorescence following electroporation with Gag replicon RNA. BHK cells were electroporated and subjected to imunofluorescence staining with an anti-Gag monoclonal antibody at 24 hours post-electroporation, to demonstrate expression of the Clade C protein.

Figure 15. Immunofluorescence detection of Du422 Gag protein expression in BHK cells. BHK cells were infected with VRP-Gag particles and subjected to immunfluorescence staining with an anti-Gag monoclonal antibody at 24 hours post-infection, to demonstrate expression of the Clade C protein.

DETAILED DESCRIPTION OF THE INVENTION

As used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" can mean a single pharmaceutical carrier or mixtures of two or more such carriers.

The present invention is based on the discovery of a vaccine for the treatment and/or prevention of infection by HIV, comprising novel combinations of isolated nucleic acids encoding two or more distinct antigens which elicit an immune response in a subject which is effective in treating and/or preventing infection by HIV. In a particular embodiment, the nucleic acids encoding the antigens of the vaccine are modified to enhance the immunogenicity of the antigen, improve the safety of the vaccine, or both.

As used herein, the term "isolated nucleic acid" means a nucleic acid separated or substantially free from at least some of the other components of the naturally occurring organism, for example, the cell structural components commonly found associated with nucleic acids in a cellular environment and/or other nucleic acids. The isolation of nucleic acids can be accomplished by well known techniques such as cell lysis or disruption of virus particles, followed by phenol plus chloroform extraction, followed by ethanol precipitation of the nucleic acids (Sambrook *et al.*, latest edition). The nucleic acids of this invention can be isolated according to methods well known in the art for isolating nucleic acids. Alternatively, the nucleic acids of the present invention can be synthesized according to standard protocols well described in the literature for synthesis, cloning and amplification of nucleic acids.

HIV-VRP Vaccines

The antigens of this invention can be gene products which are complete proteins or any fragment of a protein determined to be immunogenic by methods well known in the art. Modifications are made to the antigens of this invention to enhance immunogenicity and/or improve the safety of administration of a vaccine containing the antigen. Examples of such modifications are described in the Examples section herein. Furthermore, it is understood that, where desired, other modifications and changes (e.g., substitutions, deletions, additions) may be made in the amino acid sequence of the antigen of the present invention, which may not specifically impart enhanced immunogenicity or improved safety, yet still result in a protein or fragment which

retains all of the functional characteristics by which the protein or fragment is defined. Such changes may occur in natural isolates, may be introduced by synthesis of the protein or fragment, or may be introduced into the amino acid sequence of the protein or fragment using site-specific mutagenesis of nucleic acid encoding the protein or fragment, the procedures for which, such as mis-match polymerase chain reaction (PCR), are well known in the art.

The nucleic acids of this invention can be present in a vector and the vector of this invention can be present in a cell. The vectors and cells of this invention can be in a composition comprising the cell or vector and a pharmaceutically acceptable carrier.

The vector of this invention can be an expression vector which contains all of the genetic components required for expression of the nucleic acids of this invention in cells into which the vector has been introduced, as are well known in the art. For example, the expression vector of this invention can be a vector comprising the helper RNAs of this invention. Such an expression vector can be a commercial expression vector or it can be constructed in the laboratory according to standard molecular biology protocols. The expression vector can comprise viral nucleic acid including, but not limited to, alphavirus, flavivirus, adenovirus, retrovirus and/or adeno-associated virus nucleic acid. The nucleic acid or vector of this invention can also be in a liposome or a delivery vehicle which can be taken up by a cell via receptor-mediated or other type of endocytosis.

In another embodiment, the nucleic acids of this invention can be present in a composition comprising a population of alphavirus replicon particles which comprise two or more distinct isolated nucleic acids of this invention and wherein the nucleic acids are each contained within a separate alphavirus replicon particle (herein referred to as a "VRP"). Thus, the expression vector of the present invention can be an alphavirus replicon particle comprising a nucleic acid encoding an antigen of this invention.

In a particular embodiment, the present invention provides a composition comprising two or more isolated nucleic acids selected from the group consisting of an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of particles, e.g., virus-like particles, containing the *gag* gene product or the immunogenic fragment thereof, and their release from a cell, and an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity.

In a preferred embodiment, the invention provides alphavirus replicon particles (VRPs) that can be administered as an HIV vaccine. These HIV-VRPs are propagation defective, single cycle vector constructs that contain a self-amplifying RNA (replicon RNA), e.g., from VEE, in which the structural protein genes of the virus are replaced by a HIV-1 Clade C *gag* gene or any other HIV antigen to be expressed. Following introduction into packaging (or helper) cells *in vitro*, the replicon RNA is packaged into VRPs by supplying the viral structural proteins in *trans* (helper RNAs).

The present invention further provides a population of alphavirus replicon particles comprising two or more isolated nucleic acids selected from the group consisting of 1) an isolated nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) an isolated nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of particles, such as virus-like particles, containing the *gag* gene product or the immunogenic fragment thereof, from a cell, and 3) an isolated nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity, and

wherein the nucleic acids are each contained within a separate alphavirus replicon particle.

It is also contemplated that the compositions of this invention comprise alphavirus replicon particles in which either the replicon RNA or at least one structural protein comprises one or more attenuating mutations. Thus, the present invention additionally provides a population of alphavirus replicon particles comprising two or more distinct types of such particles selected from the group consisting of 1) particles expressing a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, 2) particles expressing a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit release of particles, such as virus-like particles, containing the *gag* gene product or the immunogenic fragment thereof, from a cell, and 3) particles expressing a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity; and wherein the nucleic acids are each contained within a separate alphavirus replicon particle and further wherein the alphavirus replicon particles comprise a replicon RNA or at least one structural protein which comprises one or more attenuating mutations.

In a preferred embodiment, the population of alphavirus replicon particles comprises particles expressing the nucleic acids encoding *pol*, *env*, and *gag* gene products. In this embodiment, vigorous antigen-specific cellular (e.g., CTL, NK cell and T-helper) and/or humoral (e.g., antibody) responses can be obtained when such particle populations are administered to a subject.

In the compositions described above, the *gag* gene product or immunogenic fragment thereof can be modified by mutation of the second codon, whereby a glycine is changed to an alanine. Alternatively, the *gag* gene product or immunogenic fragment thereof can be modified by any other means known in the art for inhibiting

the release of particles containing the *gag* gene product or immunogenic fragment thereof from a cell.

Furthermore, in the compositions of this invention, the *pol* gene product or immunogenic fragment thereof can be modified by mutation of the nucleotide sequence encoding the active site motif, whereby YMDD is changed to YMAA or HMAA (the latter providing a convenient site for cloning, see SEQ ID NO:16). The *pol* gene product or immunogenic fragment thereof can also be modified by any means known in the art for inhibiting reverse transcriptase activity.

The *pol* gene product or immunogenic fragment thereof of this invention may be further modified such that the coding sequences for protease, integrase and RNase H are removed, inactivated and/or modified, e.g., by producing only the p51 region of the *pol* gene product. This modification has been shown in some studies to reduce the possibility of formation of replication competent alphavirus particles during production of alphavirus replicon particles comprising the *pol* gene product or immunogenic fragment thereof. This modification can be of the nucleic acid encoding the *pol* gene product or immunogenic fragment thereof according to methods known in the art. Thus, the particles and compositions of this invention can comprise nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof.

In the compositions of this invention, the *gag*, *env* or *pol* gene products or immunogenic fragments thereof can be from any HIV isolate or consensus sequence derived from HIV primary isolates now known or later identified, the isolation and characterization of which are well known in the art. Also, in the compositions of this invention, the *gag*, *env* or *pol* gene products or immunogenic fragments thereof can be produced from the same HIV isolate or HIV consensus sequence or from any

combination of HIV isolates or HIV consensus sequences. In the Examples provided herein, the nucleic acid sequences encoding the *env*, *gag* and *pol* gene products of this invention were selected based on a consensus sequence generated from primary isolates obtained from recent seroconvertors in KwaZulu/Natal Province in South Africa. Sequence analysis of these isolates identified them as subtype (or clade) C, and in preferred embodiments of the invention, the *env*, *gag* and *pol* genes are from Clade C isolates of HIV.

In preferred embodiments, each of the three HIV genes are derived from one or more of the South African isolates obtained from recent seroconverters in Kwazulu/Natal as described herein (see Figures 11-13 for isolate names). In a further embodiment, the *gag* gene or gene fragment is from a *gag* sequence having 95% or greater amino acid identity with the South African consensus sequence for the *gag* gene. In a specific embodiment, the *gag* gene or fragment thereof is derived from HIV Subtype Clade C isolate Du422 and the *env* and *pol* genes or fragments thereof are derived from HIV isolate Du151.

The term "alphavirus" has its conventional meaning in the art and includes the various species of the alphavirus genus, such as Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Western Equine Encephalitis virus (WEE), Everglades virus, Mucambo virus, Pixuna virus, Sindbis virus, Semliki Forest virus, South African Arbovirus No. 86, Middleburg virus, Chikungunya virus, O-Nyong-Nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, Buggy Creek virus, as well as any specific strains of these alphaviruses (e.g., TR339; Girdwood) and any other virus classified by the International Committee on Taxonomy of Viruses (ICTV) as an alphavirus.

An "alphavirus replicon particle" as used herein is an infectious, replication defective, alphavirus particle which comprises alphavirus structural proteins and further

comprises a replicon RNA. The replicon RNA comprises nucleic acid encoding the alphavirus packaging segment, nucleic acid encoding alphavirus non-structural proteins and a heterologous nucleic acid sequence encoding an antigen of this invention. The non-structural proteins encoded by the replicon RNA may be such proteins as are required for replication and transcription. In a specific embodiment of this invention, the structure of the replicon RNA, starting at the 5' end, comprises the 5' untranslated region of the alphavirus RNA, the non-structural proteins (e.g., nsPs1-4) of the alphavirus, the 26S promoter (also known as the "subgenomic promoter"), the heterologous nucleic acid encoding an HIV antigen, and the 3' untranslated region of the alphavirus RNA. An example of a nucleic acid encoding alphavirus nonstructural proteins that can be incorporated into the embodiments of this invention is SEQ ID NO:2, which encodes the amino acid sequence of SEQ ID NO:3.

Although the alphavirus replicon RNA can comprise nucleic acid encoding one or two alphavirus structural proteins, the replicon RNA does not contain nucleic acid encoding all of the alphavirus structural proteins. The replicon RNA can lack nucleic acid encoding any alphavirus structural protein(s). Thus, the resulting alphavirus replicon particles of this invention are replication defective inasmuch as the replicon RNA does not encode all of the structural proteins required for encapsidation of the replicon RNA and assembly of an infectious virion.

As used herein, "alphavirus structural protein" or "structural protein" means the alphavirus proteins required for encapsidation of alphavirus replicon RNA and packaging of the encapsidated RNA into a virus particle. The alphavirus structural proteins include PE2, E2, E3, 6K and E1.

The alphavirus replicon particles of this invention can comprise replicon RNA from any of the alphaviruses of this invention. Furthermore, the alphavirus replicon particles of this invention can comprise alphavirus structural proteins from any of the alphaviruses of this invention. Thus, the replicon particles can be made up of replicon RNA and structural proteins from the same alphavirus or from different alphaviruses,

the latter of which would be chimeric alphavirus replicon particles (e.g., a particle comprising Sindbis virus replicon RNA and VEE structural proteins).

The alphavirus replicon particles of this invention can be made by employing a helper cell for expressing an infectious, replication defective, alphavirus particle in an alphavirus-permissive cell. The helper cell includes (a) a first helper RNA encoding (i) at least one alphavirus structural protein, and (ii) not encoding at least one alphavirus structural protein; and (b) a second helper RNA separate from the first helper RNA, the second helper RNA (i) not encoding the at least one alphavirus structural protein encoded by the first helper RNA, and (ii) encoding at least one alphavirus structural protein not encoded by the first helper RNA, such that all of the alphavirus structural proteins assemble together into alphavirus particles in the cell.

The alphavirus structural protein genes can be present on the helper RNAs of this invention in any combination. For example, the helper RNA of this invention can encode the alphavirus capsid and E1, capsid and E2, E1 and E2, capsid only, E1 only, E2 only, etc. It is also contemplated that the alphavirus structural proteins are provided in *trans* from genes located on three separate RNA molecules within the helper cell.

In a preferred embodiment, the helper cell also includes a replicon RNA, which encodes the alphavirus packaging segment and an inserted heterologous RNA. In the embodiment wherein the helper cell also includes a replicon RNA, the alphavirus packaging segment may be, and preferably is, deleted from both the first helper RNA and the second helper RNA. For example, in an embodiment wherein the helper cell includes a replicon RNA encoding the alphavirus packaging segment and an inserted heterologous RNA, the first helper RNA encodes the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, and the second helper RNA encodes the alphavirus capsid protein. In a preferred embodiment, the first helper RNA encodes the E3-E2-6k-E1cassette from an alphavirus. In an alternative embodiment, the cassette encoded on the first helper RNA is referred to as the E3-E2-E1 cassette. A specific embodiment of this aspect of the invention is diagrammed in Figure 3, and an exemplary nucleotide

sequence is SEQ ID NO:11. The replicon RNA, first helper RNA, and second helper RNA are all on separate molecules and are cotransfected, e.g., by electroporation, into the helper cell, which can be any alphavirus permissive cell, as is well known in the art.

In an alternative embodiment, the helper cell includes a replicon RNA encoding the alphavirus packaging segment and an inserted heterologous RNA and also includes the alphavirus capsid protein otherwise encoded by the second helper RNA. The first helper RNA encodes the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein. Thus, the replicon RNA and the first helper RNA are on separate molecules, and the replicon RNA and the second helper RNA are on a single molecule.

The RNA encoding the structural proteins, i.e., the first helper RNA and the second helper RNA, can include one or more attenuating mutations. In a preferred embodiment, either one or both of the first helper RNA and the second helper RNA include at least one attenuating mutation. The attenuating mutations provide the advantage that in the event of RNA-RNA recombination the resulting recombinant RNA molecules encoding the alphavirus structural and non-structural genes will yield or produce virus of decreased virulence.

The alphavirus replicon particles of this invention can be made by a) transfecting a helper cell as given above with a replication defective replicon RNA, b) producing the alphavirus particles in the transfected cell, and c) collecting the alphavirus particles from the cell. The replicon RNA encodes the alphavirus packaging segment and a heterologous RNA. The transfected helper cell further includes the first helper RNA and second helper RNA as described above.

As described hereinabove, the structural proteins used to assemble the alphavirus replicon particles of this invention are distributed among one or more helper RNAs (i.e., a first helper RNA and a second helper RNA). As noted herein, one or more structural protein genes may be located on the replicon RNA, provided that at least one structural protein gene is deleted from the replicon RNA such that the replicon

RNA and resulting alphavirus particle are replication defective. As used herein, the terms "deleted" or "deletion" mean either total deletion of the specified nucleic acid or the deletion of a sufficient portion of the specified nucleic acid to render the nucleic acid and/or its resultant gene product inoperative or nonfunctional, in accordance with standard usage. (See, e.g., U.S. Pat. No. 4,650,764 to Temin *et al.*) The term "replication defective" as used herein means that the replicon RNA cannot replicate in the host cell (i.e., produce progeny infectious viral particles) in the absence of the helper RNA. The replicon RNA is replication defective inasmuch as the replicon RNA does not include all of the alphavirus structural protein genes required for replication, at least one of the required structural protein genes being deleted therefrom.

In one embodiment, the packaging segment or "encapsidation sequence" is deleted from at least the first helper RNA. In a preferred embodiment, the packaging segment is deleted from both the first helper RNA and the second helper RNA. In a specific embodiment, the second helper RNA is constructed from a VEE cDNA clone, deleting all of the non-structural proteins (i.e., nsPs1-4) except approximately 500 nucleotides at the 5' end of nsP1, the packaging signal, and the glycoprotein cassette (E3-E2-E1). An example of a plasmid encoding such a second helper RNA is provided in Figure 2, and an exemplary nucleotide sequence for such a second helper RNA is SEQ ID NO:8.

In the preferred embodiment wherein the packaging segment is deleted from both the first helper RNA and the second helper RNA, preferably the helper cell contains a replicon RNA in addition to the first helper RNA and the second helper RNA. The replicon RNA encodes the packaging segment and an inserted heterologous RNA encoding an HIV antigen or a fragment thereof. Typically, the inserted heterologous RNA encodes a gene product which is expressed in the target cell, and includes the promoter and regulatory segments necessary for the expression of that gene product in that cell.

In another preferred embodiment, the replicon RNA, the first helper RNA and

the second helper RNA are provided on separate molecules such that a first molecule, i.e., the replicon RNA, encodes the packaging segment and the inserted heterologous RNA, a second molecule, i.e., the first helper RNA, encodes at least one but not all of the required alphavirus structural proteins, and a third molecule, i.e., the second helper RNA, encodes at least one but not all of the required alphavirus structural proteins. For example, in one preferred embodiment of the present invention, the helper cell includes a set of RNAs which include (a) a replicon RNA encoding an alphavirus packaging sequence and an inserted heterologous RNA, (b) a first helper RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein, and (c) a second helper RNA encoding the alphavirus capsid protein, so that the alphavirus E1 glycoprotein, the alphavirus E2 glycoprotein and the capsid protein assemble together into alphavirus particles containing the replicon RNA in the helper cell.

In an alternate embodiment, the replicon RNA and the first helper RNA are on separate molecules, and the replicon RNA and the second helper RNA are on a single molecule together, thereby providing a first molecule, i.e., the first helper RNA, encoding at least one but not all of the required alphavirus structural proteins, and a second molecule, i.e., the replicon RNA and second helper RNA, encoding the packaging segment, the inserted heterologous gene product and the structural protein(s) not encoded by the first helper. Thus, one or more structural protein(s) is encoded by the second helper RNA, but the second helper RNA is located on the second molecule together with the replicon RNA. For example, in one preferred embodiment of the present invention, the helper cell includes a set of RNAs including (a) a replicon RNA encoding an alphavirus packaging sequence, an inserted heterologous RNA, and an alphavirus capsid protein, and (b) a first helper RNA encoding the alphavirus E1 glycoprotein and the alphavirus E2 glycoprotein so that the alphavirus E1 glycoprotein, the alphavirus E2 glycoprotein and the capsid protein assemble together into alphavirus particles in the helper cell.

The present invention also contemplates alphavirus replicon particles which comprise replicon RNA encoding more than one heterologous gene product. For

expression of more than one heterologous nucleic acid from a single replicon RNA, a promoter can be inserted upstream of each heterologous nucleic acid on the replicon RNA, such that the promoter regulates expression of the heterologous nucleic acid, resulting in the production of more than one antigen from a single replicon RNA Another embodiment contemplates the insertion of an IRES sequence, such as the one from the picornavirus, EMC virus, between the heterologous genes downstream from a 26S promoter of the replicon, thus leading to translation of multiple antigens from a single replicon.

In one preferred embodiment of the present invention, the RNA encoding the alphavirus structural proteins, i.e., the capsid, E1 glycoprotein and/or E2 glycoprotein, contains at least one attenuating mutation. It is further contemplated that the RNA encoding the non-structural proteins can contain at least one attenuating mutation. The phrases "attenuating mutation" and "attenuating amino acid," as used herein, mean a nucleotide mutation or an amino acid coded for in view of such a mutation which result in a decreased probability of causing disease in its host (i.e., a loss of virulence), in accordance with standard terminology in the art, See, e.g., Davis *et el.* (1980). The mutation can be, for example, a substitution mutation or an in-frame deletion mutation. The phrase "attenuating mutation" excludes mutations which would be lethal to the virus. Thus, according to this embodiment, the E1 RNA and/or the E2 RNA and/or the capsid RNA can include at least one attenuating mutation. In a more preferred embodiment, the E1 RNA and/or the E2 RNA and/or the capsid RNA includes at least two, or multiple, attenuating mutations. The multiple attenuating mutations may be positioned in either the first helper RNA or in the second helper RNA, or they may be distributed randomly with one or more attenuating mutations being positioned in the first helper RNA and one or more attenuating mutations positioned in the second helper RNA. Appropriate attenuating mutations will be dependent upon the alphavirus used, as is well known in the art.

For example, when the alphavirus is VEE, suitable attenuating mutations can be in codons at E2 amino acid position 76 which specify an attenuating amino acid,

preferably lysine, arginine, or histidine as E2 amino acid 76; codons at E2 amino acid position 120 which specify an attenuating amino acid, preferably lysine as E2 amino acid 120; codons at E2 amino acid position 209 which specify an attenuating amino acid, preferably lysine, arginine, or histidine as E2 amino acid 209; codons at E1 amino acid 272 which specify an attenuating mutation, preferably threonine or serine as E1 amino acid 272; codons at E1 amino acid 81 which specify an attenuating mutation, preferably isoleucine or leucine as E1 amino acid 81; and codons at E1 amino acid 253 which specify an attenuating mutation, preferably serine or threoinine as E1 amino acid 253; and the combination mutation of the deletion of E3 codons 56-59 together with codons at E1 amino acid 253 which specify an attenuating mutation, as provided herein. Other suitable attenuating mutations within the VEE genome will be known to those skilled in the art.

In an alternate embodiment, wherein the alphavirus is the South African Arbovirus No. 86 (S.A.A.R.86), suitable attenuating mutations can be, for example, in codons at nsP1 amino acid position 538 which specify an attenuating amino acid, preferably isoleucine as nsP1 amino acid 538; codons at E2 amino acid position 304 which specify an attenuating amino acid, preferably threonine as E2 amino acid 304; codons at E2 amino acid position 314 which specify an attenuating amino acid, preferably lysine as E2 amino acid 314; codons at E2 amino acid position 376 which specify an attenuating amino acid, preferably alanine as E2 amino acid 376; codons at E2 amino acid position 372 which specify an attenuating amino acid, preferably leucine as E2 amino acid 372; codons at nsP2 amino acid position 96 which specify an attenuating amino acid, preferably glycine as nsP2 amino acid 96; codons at nsP2 amino acid position 372 which specify an attenuating amino acid, preferably valine as nsP2 amino acid 372; in combination, codons at E2 amino acid residues 304, 314, 372 and 376; codons at E2 amino acid position 378 which specify an attenuating amino acid, preferably leucine as E2 amino acid 378; codons at nsP2 amino acid residue 372 which specify an attenuating mutation, preferably valine as nsP2 amino acid 372; in combination, codons at nsP2 amino acid residues 96 and 372 attenuating substitution mutations at nsP2 amino acid residues 96 and 372; codons at nsP2 amino acid residue

529 which specify an attenuating mutation, preferably leucine, at nsP2 amino acid residue 529; codons at nsP2 amino acid residue 571 which specify an attenuating mutation, preferably asparagine, at nsP2 amino acid residue 571; codons at nsP2 amino acid residue 682 which specify an attenuating mutation, preferably arginine, at nsP2 amino acid residue 682; codons at nsP2 amino acid residue 804 which specify an attenuating mutation, preferably arginine, at nsP2 amino acid residue 804; codons at nsP3 amino acid residue 22 which specify an attenuating mutation, preferably arginine, at nsP3 amino acid residue 22; and in combination, codons at nsP2 amino acid residues 529, 571, 682 and 804, and at nsP3 amino acid residue 22, specifying attenuating amino acids at nsP2 amino acid residues 529, 571, 682 and 804 and at nsP3 amino acid residue 22. Other suitable attenuating mutations within the S.A.A.R.86 genome will be known to those skilled in the art.

The alphavirus capsid gene used to make alphavirus replicon particles can also be subjected to site-directed mutagenesis. The altered capsid protein provides additional assurance that recombination to produce the virulent virus will not occur. The altered capsid protein gene which functions in particle assembly but not in autoproteolysis provides helper function for production of replicon particles, but does not allow for production of a viable recombinant. The capsid residues required for proteolytic function are known (Strauss *et al.,* 1990).

Suitable attenuating mutations useful in embodiments wherein any of the alphaviruses of this invention are employed are known to or can be identified by those skilled in the art using routine protocols. Attenuating mutations may be introduced into the RNA by performing site-directed mutagenesis on the cDNA which encodes the RNA, in accordance with known procedures. See Kunkel (1985), the disclosure of which is incorporated herein by reference in its entirety. Alternatively, mutations may be introduced into the RNA by replacement of homologous restriction fragments in the cDNA which encodes for the RNA, in accordance with known procedures. The identification of a particular mutation in an alphavirus as attenuating is done using routine experimentation according to methods well known in the art.

Preferably, the helper RNA of this invention includes a promoter. It is also preferred that the replicon RNA includes a promoter. Suitable promoters for inclusion in the helper RNA and replicon RNA are well known in the art. One preferred promoter is the alphavirus 26S promoter, although many suitable promoters are available, as is well known in the art.

In the system wherein a first helper RNA, a second helper RNA, and a replicon RNA are all on separate molecules, if the same promoter is used for all three RNAs, then a homologous sequence between the three molecules is provided. Thus, it is advantageous to employ different promoters on the first and second helper RNAs to provide further impediment to RNA recombination that might produce virulent virus. It is preferred that the selected promoter is operative with the non-structural proteins encoded by the replicon RNA molecule.

The infectious, replication defective, alphavirus particles of this invention are prepared according to the methods disclosed herein in combination with techniques known to those skilled in the art. The methods include, for example, transfecting an alphavirus-permissive cell with a replication defective replicon RNA including the alphavirus packaging segment and an inserted heterologous RNA, a first helper RNA encoding at least one alphavirus structural protein, and a second helper RNA encoding at least one alphavirus structural protein which is different from that encoded by the first helper RNA; producing the alphavirus particles in the transfected cell; and collecting the alphavirus particles from the cell.

Methods for transfecting the alphavirus-permissive cell with the replicon RNA and helper RNAs can be achieved, for example, by (i) treating the cells with DEAE-dextran, (ii) by lipofection, by treating the cells with, for example, LIPOFECTIN, and (iii) by electroporation, with electroporation being a preferred means of achieving RNA uptake into the alphavirus-permissive cells. Examples of these techniques are well known in the art, see e.g., U.S. Pat. No. 5,185,440 to Davis *et al.*, and PCT Publication No. WO 92/10578 to Bioption AB, the disclosures of which

are incorporated herein by reference in their entirety.

The steps of producing the infectious viral particles in the cells may also be carried out using conventional techniques. See e.g., U.S. Patent No. 5,185,440 to Davis *et al.*, PCT Publication No. WO 92/10578 to Bioption AB, and U.S. Patent No. 4,650,764 to Temin *et al.* (although Temin *et al.*, relates to retroviruses rather than alphaviruses). The infectious viral particles may be produced by standard cell culture growth techniques.

The steps of collecting the infectious alphavirus particles may also be carried out using conventional techniques. For example, the infectious particles may be collected by cell lysis, or collection of the supernatant of the cell culture, as is known in the art. See e.g., U.S. Patent No. 5,185,440 to Davis *et al.*, PCT Publication No. WO 92/10578 to Bioption AB, and U.S. Patent No. 4,650,764 to Temin *et al.* (although Temin *et al.* relates to retroviruses rather than alphaviruses). Other suitable techniques will be known to those skilled in the art. Optionally, the collected infectious alphavirus particles may be purified, if desired. Purification techniques for viruses are well known to those skilled in the art, and these are suitable for the purification of small batches of infectious alphavirus particles.

Thus, the present invention provides a method of making the populations of alphavirus replicon particles of this invention comprising:

A) (a) providing a first helper cell for producing a first population of infectious, defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and

furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

B) (a) providing a second helper cell for producing a second population of infectious, defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit formation of virus-like particles containing the *gag* gene product or the immunogenic fragment thereof and their release from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional

helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

C) providing a third helper cell for producing a third population of infectious, defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity or is modified to inactivate or delete protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the populations of alphavirus replicon particles.

In a preferred embodiment, as noted above, the method provided also includes a mutation in the *pol* gene product or immunogenic fragment thereof resulting in inactivation or deletion of protease, integrase and RNase H functions of the *pol* gene product or immunogenic fragment thereof. In a specific embodiment of this method, the region of the *pol* gene encoding the protease, RNase H and integrase function of the *pol* gene product or immunogenic fragment thereof has been deleted.

A method of making the populations of alphavirus replicon particles of this invention, wherein the particles comprise at least one attenuating mutation, is also provided, comprising:

A) (a) providing a first helper cell for producing a first population of infectious, defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the first population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

B) providing a second helper cell for producing a second population of infectious, defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *gag* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit release of particles, such as virus-like particles, containing the *gag* gene product or the immunogenic fragment thereof from a cell, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and

furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the second population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells;

C) providing a third helper cell for producing a third population of infectious, defective alphavirus particles, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to inhibit reverse transcriptase activity or is modified to inactivate or delete protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein;

and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the third population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture, and further wherein at least one of said replicon RNA, said first helper RNA, and said one or more additional helper RNA(s) comprises one or more attenuating mutations;

(b) producing the alphavirus particles in the helper cell; and

(c) collecting the alphavirus particles from the helper cells; and

D) combining the first population of alphavirus particles produced from the first helper cell, the second population of alphavirus particles produced from the second helper cell and the third population of alphavirus particles produced from the third helper cell, thereby producing the populations of alphavirus replicon particles of the present invention comprising at least one attenuating mutation.

In a preferred embodiment, as noted above, the method provided above can include a further mutation in the *pol* gene product or immunogenic fragment thereof resulting in inactivation or deletion of protease, integrase and RNase H functions of the *pol* gene product or immunogenic fragment thereof. In a specific embodiment of this method, the region of the *pol* gene encoding the protease, RNase H and integrase function of the *pol* gene product or immunogenic fragment thereof has been deleted.

It is also contemplated regarding the method described above, that not all of the first, second and third populations of alphavirus particles do not all have to comprise an

attenuating mutation. For example, the first population may comprise attenuating mutations, but the second and third populations may not, etc.

The present invention further provides the compositions of the present invention which are produced by the methods of this invention.

The compositions and methods of this invention which incorporate attenuating mutations into the alphavirus replicon particles forming the composition and/or produced by the methods include purified compositions and methods of purification based on the presence of the attenuating mutations. In particular, certain attenuating mutations in the alphavirus structural proteins introduce heparin binding sites into these proteins which are present on the surface of the alphavirus replicon particles. As an example, the V3014 E2 glycoprotein (SEQ ID NO:12 and SEQ ID NO:13) has a mutation in which a lysine is substituted for the glutamic acid at amino acid position 209. This mutation, which creates a more positively charged glycoprotein, increases the affinity of this protein for heparin. Thus, it is possible to purify such particles using heparin affinity chromatography. Such chromatography can be performed using any of several commercially available resins to which heparin has been bound. The source of heparin is variable; the commercially available resins currently use porcine heparin. The choice of resin will be based on its relative ease of use in a scaled-up, GMP-compliant process, e.g., price, column packing limitations, and potential for easy sanitization. The use of heparin affinity chromatography results in a substantial purification of the VRPs with very little loss of material, and it is a scalable purification step. In a preferred embodiment, a heparin affinity chromatography step results in between an 8- to 27-fold reduction in total protein per ml, or from a 300- to 1000-fold reduction in total protein per VRP. Thus, the present invention provides heparin affinity-purified alphavirus replicon particles containing attenuating mutations which are useful as clinical trial material and commercial product. The present invention also provides methods for preparing purified alphavirus replicon particles containing attenuating mutations comprising the use of heparin affinity chromatography, as described in the Examples provided herein. These particles can also be present in a

composition of this invention.

The alphavirus replicon particles of this invention can also be made in a cell free system. Such replicon particles are herein referred to as virosomes. In a specific embodiment of the method, such particles are constructed from a mixture containing replicon RNA that does not encode all of the alphavirus structural proteins, purified glycoproteins E1 and E2, one or more non-cationic lipids, such as lecithin, and detergent. Detergent is slowly removed from the mixture to allow formation of lipid bilayers with incorporated RNA and glycoproteins.

In preferred embodiments of the methods of this invention, the glycoproteins E1 and E2 could be expressed in any recombinant protein expression system capable of glycosylation of mammalian proteins, such as stably transformed cell lines, for example CHO cells, or viral vector expression systems such as vaccinia, baculovirus, herpes virus, alphavirus or adenovirus. In a preferred embodiment, following expression of the proteins, the E1 and E2 glycoproteins are purified from contaminating cellular proteins in the expression supernatant. The purification of these glycoproteins can be achieved by affinity chromatographic column purification, for example using lectin-, heparin- , or antibody-affinity columns. This affinity purification step may be preceded by selective precipitation or selective extraction from the expression system supernatant by methods including, but not limited to, ammonium sulfate precipitation or detergent extraction respectively. Final polishing steps of purification may include ion-exchange chromatography or buffer exchange, for example, and tangential flow methods to generate purified glycoproteins suitable for virosome assembly.

Thus, the present invention provides a method of producing alphavirus replicon virosomes, comprising: a) combining alphavirus replicon RNA, alphavirus glycoproteins E1 and E2, non-cationic lipids and detergent; and b) gradually removing detergent, whereby alphavirus replicon virosomes are produced. This method is described in more detail in the Examples section herein.

The present invention also provides alphavirus replicon virosomes comprising an alphavirus replicon RNA encapsidated by a lipid bilayer in which alphavirus glycoproteins are embedded. The replicon RNA can be from any alphavirus and the glycoproteins can be from any alphavirus. In a specific embodiment, the alphavirus glycoproteins are VEE E1 and E2. The advantage of the alphavirus replicon virosomes is the ease of preparation, their stability, and their purity, since they are devoid of any cellular components being made in a cell free system.

The helper cells, RNAs and methods of the present invention are useful in *in vitro* expression systems, wherein the inserted heterologous RNA located on the replicon RNA encodes a protein or peptide which is desirably produced *in vitro*. The helper cells, RNAs, methods, compositions and pharmaceutical formulations of the present invention are additionally useful in a method of administering a protein or peptide to a subject in need of the desired protein or peptide, as a method of treatment or otherwise.

It is contemplated that the proteins, peptides, nucleic acids, vectors and alphavirus replicon particles of this invention can be administered to a subject to impart a therapeutic or beneficial effect. Therefore, the proteins, peptides, nucleic acids, vectors and particles of this invention can be present in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject, along with the nucleic acid or vector of this invention, without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art (see, e.g., *Remington's Pharmaceutical Science*; latest edition).

Pharmaceutical formulations of this invention, such as vaccines, of the present invention can comprise an immunogenic amount of the alphavirus replicon particles as

disclosed herein in combination with a pharmaceutically acceptable carrier. An "immunogenic amount" is an amount of the infectious alphavirus particles which is sufficient to evoke an immune response (humoral and/or cellular immune response) in the subject to which the pharmaceutical formulation is administered. An amount of from about 10^3 to about 10^7 replicon-containing particles, and preferably, about 10^4 to about 10^6 replicon-containing particles per dose is believed suitable, depending upon the age and species of the subject being treated. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution.

Subjects which may be administered immunogenic amounts of the infectious, replication defective alphavirus particles of the present invention include, but are not limited to, human and animal (e.g., horse, donkey, mouse, hamster, monkey) subjects. Administration may be by any suitable means, such as intraperitoneal or intramuscular injection.

Pharmaceutical formulations for the present invention can include those suitable for parenteral (e.g., subcutaneous, intradermal, intramuscular, intravenous and intraarticular) administration. Alternatively, pharmaceutical formulations of the present invention may be suitable for administration to the mucous membranes of a subject (e.g., intranasal administration). The formulations may be conveniently prepared in unit dosage form and may be prepared by any of the methods well known in the art.

Thus, the present invention provides a method for delivering nucleic acids and vectors (e.g., alphavirus replicon particles; virosomes) encoding the antigens of this invention to a cell, comprising administering the nucleic acids or vectors to a cell under conditions whereby the nucleic acids are expressed, thereby delivering the antigens of this invention to the cell. The nucleic acids can be delivered as naked DNA or in a vector (which can be a viral vector) or other delivery vehicles and can be delivered to cells *in vivo* and/or *ex vivo* by a variety of mechanisms well known in the art (e.g., uptake of naked DNA, viral infection, liposome fusion, endocytosis and the like). The

cell can be any cell which can take up and express exogenous nucleic acids.

Further provided herein is a method of inducing an immune response to an HIV antigen of this invention in a subject, comprising administering to the subject an immunogenic amount of the particles, virosomes and/or composition of this invention, in a pharmaceutically acceptable carrier.

A method of treating and/or preventing infection by HIV in a subject is also provided herein, comprising administering to the subject an effective amount of the particles, virosomes and/or compositions of this invention, in a pharmaceutically acceptable carrier.

The subject of this invention can be any animal in which an immune response can be induced or in which an infection by HIV can be treated and/or prevented. In a preferred embodiment, the subject of this invention is a mammal and most preferably is a human.

Protocols and data regarding the testing of the compositions of this invention in animals and protocols for administration to humans are provided in the Examples herein.

In a particular embodiment, the present invention provides an isolated nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the protease, integrase, RNase H and reverse transcriptase functions of the *pol* gene product or immunogenic fragment thereof have been inactivated or deleted. Such a modification has been shown in some studies to facilitate inhibition of the formation of replication competent alphavirus particles during production of alphavirus replicon particles comprising the *pol* gene product or immunogenic fragment thereof.

Also provided herein is a composition comprising the *pol*-expressing nucleic

acid described above, a vector comprising the nucleic acid and a cell comprising the vector. The *pol*-expressing nucleic acid can also be present in an alphavirus replicon particle comprising the nucleic acid.

As noted above, the nucleic acid encoding the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in the inhibition of reverse transcriptase activity. In a preferred embodiment, a mutation is introduced at the active site motif that results in inhibition of reverse transcriptase activity. Such a mutation may remove the DNA binding domain of the enzyme, for example. A mutation from YMDD to YMAA or HMAA at this motif is an example of such a mutation.

The present invention additionally provides a method of making an alphavirus replicon particle comprising nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions from the *pol* gene product or immunogenic fragment thereof, comprising

A) providing a helper cell for producing an infectious, defective alphavirus particle, comprising in an alphavirus-permissive cell:

(i) an alphavirus replicon RNA, wherein the replicon RNA comprises an alphavirus packaging signal and a nucleic acid encoding a *pol* gene product or an immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof is modified to delete or inactivate protease, RNase H, integrase and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, and wherein the replicon RNA lacks sequences encoding alphavirus structural proteins;

(ii) a first helper RNA separate from said replicon RNA, said first helper RNA encoding at least one alphavirus structural protein and furthermore not encoding at least one other alphavirus structural protein; and

(iii) one or more additional helper RNA(s) separate from said replicon RNA and separate from said first helper RNA, said additional helper RNA(s) encoding at least one other alphavirus structural protein not encoded by said first helper RNA;

and with at least one of said helper RNAs lacking an alphavirus packaging signal;

wherein the combined expression of the alphavirus replicon RNA and the helper RNAs produces an assembled alphavirus particle which is able to infect a cell, and is unable to complete viral replication, and further wherein the population contains no detectable replication-competent alphavirus particles as determined by passage on permissive cells in culture;

(B) producing the alphavirus particles in the helper cell; and

(C) collecting the alphavirus particles from the helper cell.

In the method provided above, at least one of the replicon RNA, the first helper RNA, and the one or more additional helper RNA(s) can comprise one or more attenuating mutations, as described herein.

In a specific embodiment of this method, a mutation is introduced at the active site motif in the *pol* gene product or immunogenic fragment thereof that results in inhibition of reverse transcriptase activity. Such a mutation may remove the DNA binding domain of the enzyme, for example. A mutation from YMDD to YMAA or HMAA at this motif is an example of such a mutation.

Also provided herein is an alphavirus replicon particle expressing the *pol* gene product or immunogenic fragment thereof, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, produced according to any of the above methods.

In a further embodiment, the present invention provides a method of inducing an immune response in a subject, comprising administering to the subject an immunogenic amount of a composition comprising an alphavirus particle comprising nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, in a pharmaceutically acceptable carrier.

Furthermore, the present invention provides a method of treating or preventing infection by human immunodeficiency virus in a subject, comprising administering to the subject an effective amount of a composition comprising an alphavirus particle comprising nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in deletion or inactivation of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, in a pharmaceutically acceptable carrier.

In preferred embodiments of the methods of this invention, the subject is administered an effective amount of a population of alphavirus particles comprising particles expressing (1) nucleic acid encoding a *pol* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *pol* gene product or immunogenic fragment thereof comprises a modification resulting in inactivation or deletion of protease, integrase, RNase H and reverse transcriptase functions in the *pol* gene product or immunogenic fragment thereof, (2) nucleic acid encoding a *gag* gene product or immunogenic fragment thereof of a human immunodeficiency virus, wherein the *gag* gene product or immunogenic fragment thereof is modified to inhibit release of *gag* gene product or the immunogenic fragment thereof from a cell, and (3) nucleic acid encoding an *env* gene product or an immunogenic fragment thereof of a human immunodeficiency virus in a pharmaceutically acceptable carrier.

In further preferred embodiments, the population of alphavirus particles comprises particles expressing (1) nucleic acid encoding a *gag* gene sequence that has at least 95% identity with SEQ ID NO:4; (2) nucleic acid encoding a *pol* gene sequence that has at least 99% identity with SEQ ID NO:15; and (3) nucleic acid encoding an *env* gene sequence with at least 92% identity with SEQ ID NO:18. In a specific embodiment, the population of alphavirus particles comprises particles expressing (1) nucleic acid of SEQ ID NO:4, (2) nucleic acid of SEQ ID NO:15, and (3) nucleic acid of SEQ ID NO:18.

EXAMPLES

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In these examples, nm means nanometer, mL means milliliter, pfu/mL means plaque forming units/milliliter, VEE means Venezuelan Equine Encephalitis virus, EMC means encephalomyocarditis virus, BHK means baby hamster kidney cells, HA means hemagglutinin gene, N means nucleocapsid, FACS means fluorescence activated cell sorter, and IRES means internal ribosome entry site. The expression "E2 amino acid (e.g., lys, thr, etc.) number" indicates the designated amino acid at the designated residue of the E2 gene, and is also used to refer to amino acids at specific residues in the E1 protein and in the E3 protein, respectively.

EXAMPLE 1

VEE Replicon Particles as Vaccines

Replicon particles for use as a vaccine are produced using the VEE-based vector system, originally developed from a full-length, infectious cDNA clone of the RNA genome of VEE (Figure 1 in Davis *et al.*, 1989). In this Example, one or more attenuating mutations (Johnston and Smith, 1988; Davis *et al.*, 1990) have been inserted into the clone to generate attenuated VEE vaccine vectors (Davis *et al.*, 1991; 1995; Grieder *et al.*, 1995).

As described herein, these constructs are genetically modified to create an RNA replicon (i.e., an RNA that self-amplifies and expresses), and one or more helper RNAs to allow packaging. The replicon RNA expresses an HIV gene, e.g., the Clade C HIV-1 *gag* gene. The replicon RNA is packaged into virus-like particles (herein referred to as "virus replicon particles" or "VRPs") that are infectious for only one cycle. During this cycle, the characteristics of the alphavirus-based vector result in very high levels of expression of the replicon RNA in cells to which the VRP is targeted, e.g., cells of the lymph node.

In the cytoplasm of the target cell, the replicon RNA is first translated to produce the viral replicase proteins necessary to initiate self-amplification and expression. In this Example, the HIV-1 Clade C *gag* gene is encoded by a subgenomic mRNA, abundantly transcribed from a negative-sense replicon RNA intermediate, leading to high-level expression of the HIV-1 Clade C *gag* gene product. Since the VEE structural protein genes are not encoded by the replicon RNA, progeny virion particles are not assembled, thus limiting the replication to a single cycle within the infected target cell.

Importantly, only the replicon RNA is packaged into VRPs, as the helper RNAs lack the *cis*-acting packaging sequence required for encapsidation. The "split helper" or bipartite system (see Example 4) greatly reduces the chance for an intact genome being assembled by recombination, and as a back-up safety feature, one or more highly attenuating mutations, such as those contained in the glycoprotein genes in V3014 (Grieder *et al.*, 1995), are incorporated.

Overall, the design of the VRPs incorporates several layered and redundant safety features. In addition to the above-described split helper system and attenuating mutations, over one-third of the genome of the virus has been removed, creating a defective genome which prevents spread from the initially infected target cell. Nonetheless, if a statistically rare recombination event occurs to yield replication

competent virus (RCV), the resulting virus would be a highly attenuated VEE strain.

EXAMPLE 2

Construction of VEE Replicon

The VEE structural protein genes (C-PE2-6K-E1) are removed from a cDNA clone pV3014 which contained two attenuating mutations (E2 lys 209, E1 thr 272), and a duplication of the 26S subgenomic RNA promoter sequence immediately downstream from the 3'-end of the E1 glycoprotein gene, followed by a multiple cloning site as described in U.S. Pat. No. 5,505,947 to Johnston *et al.* The pV3014 plasmid DNA is digested to completion with ApaI restriction enzyme, which cuts the VEE genomic sequence at nucleotide 7505 (numbered from the 5'-end of the genome sequence). A second recognition site for this enzyme is found in the duplicate 26S subgenomic promoter. Therefore, digestion of pV3014 with ApaI produces two DNA fragments, one containing the VEE nonstructural genes (e.g., SEQ ID NO:2) and a single copy of the 26S subgenomic RNA promoter followed by a multiple cloning site, and a second smaller fragment containing a 26S subgenomic RNA promoter followed by the VEE structural genes. The large fragment is isolated and religated to produce the replicon, pVR2. A multiple cloning site (MCS) was inserted into pVR2 to generate pVERV. In this example, as well as in the construction of the helper plasmids (Example 3), the ampicillin resistance gene in each plasmid was replaced with a kanamycin resistance gene (SEQ ID NO:6; encoding amino acid sequence as in SEQ ID NO:7). The kanamycin resistance gene was obtained from the pET-9a plasmid, and was used to aid in the cloning manipulations and for regulatory compliance.

EXAMPLE 3

Construction of Helper Plasmids

The starting materials for the helper plasmids are four full-length cDNA clones: V3000, the virulent Trinidad donkey strain of VEE, three clones with attenuating mutations, pV3014 (E2 lys 209, E1 thr 272), V3519 (E2 lys 76, E2 lys 209, E1 thr 272)

and V3526 (deletion of E3 56-59, E1 ser 253), which are in the genetic background of Trinidad donkey strain VEE. Several different helper plasmids have been made by using unique or rare restriction sites in the full-length cDNA clone to delete portions of the nonstructural protein region. The full-length clone is digested with one or two restriction enzymes, the larger DNA fragment is isolated and then religated to form a functional plasmid. *In vitro* RNA transcripts from these plasmids upon transfection of tissue culture cells would not encode a functional RNA replication complex, and also would not include an encapsidation signal. The helper constructs differ in the size of the nonstructural gene deletion. The helper constructs are designated by the attenuated mutant clone used in their construction, and by the percentage of the nonstructural region deleted. The following helper constructs were generated:

V3014Δ520-7507(93%)
V3519Δ520-7507(93%)
V3526Δ520- 7505(93%)
V3014Δ520-6965(87%)
V3519Δ1687-7507(78%)
V3014Δ2311-7505(70%)
V3519Δ3958-7507(47%)
V3526Δ520-7505(93%)
V3014Δ3958-7505(47%)
V3519Δ1955-3359(19%)
V3014Δ520-3954(46%)
V3014Δ1955-3359(19%)
V3014Δ1951-3359(19%)
V3014Δ2311-3055(10%)
V3014Δ2307-3055(10%)

EXAMPLE 4

Construction of Bipartite RNA Helper Plasmids

A bipartite helper system is constructed as described herein. The V3014Δ520-7505(93%) helper is used to construct an additional deletion of the E2 and E1 glycoprotein genes by digestion with HpaI restriction enzyme and ligation, resulting in deletion of the sequence between nucleotide 8494 (in the E3 gene) and nucleotide 11,299 (near the 3'-end of the E1 gene). *In vitro* RNA transcripts of this glycoprotein helper plasmid (presented graphically in Figure 2; an exemplary nucleotide sequence for such a plasmid is SEQ ID NO:8, including the nucleotide sequence (SEQ ID NO:9 and the amino acid sequence (SEQ ID NO:10 of the VEE capsid), when electroporated into BHK cells with a replicon RNA, are replicated and transcribed to give a mRNA encoding only the capsid protein of VEE.

The second member of the bipartite helper is constructed from the same original helper plasmid 3014Δ5207505(93%) by cleavage with Tth111I restriction enzyme (at nucleotide 7544) and SpeI restriction enzyme (at nucleotide 8389), resulting in deletion of the capsid gene, followed by insertion of a synthetic double-stranded oligonucleotide with Tth111I and SpeI termini. The inserted sequence restored the downstream portion of the 26S promoter and an ATG initiation codon followed by a Ser codon, such that the first amino acid residue of E3 (Ser) is the first codon following the inserted AUG. The resulting glycoprotein helper plasmid is presented graphically in Figure 3, and an exemplary nucleic acid sequence for such a plasmid is SEQ ID NO:11, encoding the VEE glycoproteins (E3-E2-6kD-E1), SEQ ID NO:12. The *in vitro* transcript of this plasmid, when transfected into a cell with replicon RNA, will produce the VEE glycoproteins (SEQ ID NO:13). Co-electroporation of both of these helper RNAs into a cell with replicon RNA results in production of infectious particles containing only replicon RNA.

Other than the 5' and 3' ends and the 26S promoters (40 nucleotides) of these helper RNAs, the only sequence in common between the capsid and glycoprotein helpers is the sequence from 8389 to 8494 (106 nucleotides)

EXAMPLE 5

VEE REPLICON PARTICLES EXPRESSING HIV GENES

The vaccines of this invention are exemplified by the use of a propagation defective, replicon particle vector system derived from an attenuated strain of Venezuelan equine encephalitis virus (VEE) to create a mixture of VEE replicon particles individually expressing HIV-1 *gag*, *pol*, or *env* genes. The three genes used in this Example were selected based on homology to consensus sequences generated from primary isolates obtained from recent seroconverters in KwaZulu/Natal Province, South Africa. Plasma samples from approximately 20 recent seroconverters in the Durban/Hlabisa cohort and a similar number of HIV-positive, asymptomatic individuals were collected. HIV viral RNA was isolated from the plasma, and the sequences of the *gag*, *pol* and *env* genes were analyzed. Two regions from each gene were amplified, and the resulting PCR products were sequenced (see Figure 10 for regions analyzed). A consensus sequence was derived for each gene, and the sequences of each isolate were compared to the derived consensus. All isolates were found to be Subtype C of HIV, thus confirming the predominance of this subtype in South Africa.

A. CONSTRUCTION OF THE Gag-VRP VACCINE

Described herein is the design and manufacture of VEE replicon particles (VRPs) engineered to express the *gag* gene from a Subtype C isolate of HIV-1. The main purpose of this single antigen vaccine is to establish a safety profile for VRPs in healthy human subjects. Optimally, the HIV-Gag-VRPs will be formulated as a component of a trivalent vaccine, also containing HIV-Pol-VRP and HIV-gp160-VRP (*env*) made in analogous procedures to the one described herein for HIV-Gag-VRPs.

In this Example, the VEE particles are based on the V3014 glycoprotein helper plasmid (Figure 3, SEQ ID NO:12 and SEQ ID NO:13), which harbors two highly attenuating mutations, one in E2 and the other in E1 (Grieder *et al.*, 1995). The V3014 glycoprotein helper RNA is able to package VRPs with significantly greater efficiency

than the glycoprotein helper RNA derived from V3526 (Pushko *et al.*, 1997). Nonetheless, safety of the VRP vector system has not been compromised since detailed pathogenesis studies clearly have shown V3014 to be avirulent in adult mice by subcutaneous inoculation (Grieder *et al.*, 1995). V3014 was found to be significantly impaired in its ability to reach and spread beyond the draining lymph node following subcutaneous inoculation. Unlike wild-type V3000, V3014 does not establish a viremia and does not reach the brain. In addition, on rare occasions when found, histopathological lesions in the periphery were much less severe than those induced by wild-type V3000 (Grieder *et al.*, 1995). Following inoculation with V3014, adult mice are protected against lethal wild-type VEE infection.

The attenuated phenotype of V3014 also was observed in VEE challenge studies in horses. Animals inoculated subcutaneously with V3014 showed no significant leukopenia or febrile response compared to mock-vaccinated controls. In addition, results indicated that these animals were completely protected against virulent VEE (V3000) challenge.

Taken together, these data indicate that if the rare recombination event did occur during VRP assembly to yield RCV, the worst case scenario would be the generation of a highly attenuated strain of VEE.

B. SELECTION AND CLONING OF THE HETEROLOGOUS ANTIGEN

The exemplary HIV genes used in this invention, *gag*, *pol* and *env*, are derived from Subtype C (Clade C) viruses isolated from likely Phase III clinical trial sites in South Africa. The HIV infection rate in South Africa and its long established virology and public health infrastructure make this country an attractive choice for clinical testing of HIV vaccines. Focused sequencing and phylogenetic analysis of the *gag*, *pol*, and *env* genes of these isolates has allowed the selection of genes representative of the Clade C isolates circulating in this region of Africa.

1. HIV-1 Clade C *gag* gene

Two 400 bp regions of the *gag* gene were sequenced from approximately 30 plasma samples collected from HIV seropositive individuals in South Africa. A South African consensus sequence was then determined for the *gag* gene as well as a consensus sequence from the Los Alamos database for Subtype C virus. In addition, approximately 20 comparable sequences from Malawi were used, generated as part of another study, to confirm conclusions about sequence variation. Several isolates that were close to the South African consensus sequence were compared to other isolates in distance measurements. Among these 30 isolates, one was chosen as the source for the *gag* gene (SEQ ID NO:4; corresponding to the amino acid sequence in SEQ ID NO:5) for the following reasons.

This isolate had greater than 95% amino acid identity to the South African consensus sequence, representing the approximate middle of the sequence diversity of all isolates. This isolate, known as Du422, came from a recent seroconvertor, reflecting currently circulating strains and the transmitted phenotype. The phenotype of Du422 is NS1, CCR5(+), and CXCR4(-).

Prior to the insertion of the *gag* gene into the VEE replicon plasmid vector, the amino terminal myristylation ("myr") site of *gag* was removed to prevent the formation of Gag-containing virus-like particles. Restriction enzyme digests of the *gag* gene plasmid, the capsid helper plasmid, and the glycoprotein helper plasmid were performed to confirm the identity of the three vectors when compared to published maps of the parental plasmid pBR322, with the kanamycin resistance gene substituted for the ampicillin resistance gene. The confirmed plasmid maps of the VEE replicon plasmid containing the Du422 *gag* gene (p3-40.1.6), the capsid helper plasmid (p3-13.2.2), and the glycoprotein helper plasmid (p3-13.4.6) are presented in Figures 1, 2, and 3, respectively. The full nucleotide sequence of each of these plasmids is presented herein as SEQ ID NO:1, SEQ ID NO:8, and SEQ ID NO:11, respectively.

In Figures 6 and 15, expression of this HIV-1 Gag protein in BHK cells infected

with VRPs expressing such a *gag* construct is demonstrated (Figure 6: Western blot, lane 3; Figure 15, immunofluorescence detection). The cells were infected at a multiplicity of infection (m.o.i.) of 3.5 infectious units (i.u.) per cell, and expression was measured 18 hours post-infection (p.i.). Cell lysates (from approximately 2 x 10^3 cells) were collected and fractionated either by a 4-12% gradient SDS-PAGE or by 10% SDS-PAGE. The fractionated polypeptides were transferred to PVDF membranes and probed with human HIV-1 positive serum.

2. HIV-1 Clade C *env* gene

A Clade C *env* gene (aka "gp160") from another HIV isolate, Du151, from a recent seroconverter was chosen based on its 92% amino acid identity to the South African consensus sequence for this gene, determined in an analogous method to the one described for the *gag* gene in Example 5.A.1. The phenotype of the Du151 isolate is NS1, CCR5(+), CXCR4(-). This gene was engineered into a VEE RNA replicon plasmid as shown in Figure 5, and the entire sequence of the plasmid is given at SEQ ID NO:17. The *env* gene construct used in this Example is SEQ ID NO:18.

In Figure 6, expression of this ENV protein (SEQ. ID. NO:19) in BHK cells infected with VRPs expressing this HIV *env* construct is demonstrated (Western blot, lane 2), showing that the protein expressed in the cells is of the correct size and is immunoreactive. In Figure 7, expression of this ENV protein in U87.CD4.CCR5 cells is shown. These cells process the ENV protein into two components, gp120, gp41 and gp160. In these cells, the expressed gp160 is fusogenic (see Figure 8).

3. HIV-1 Clade C *pol* gene

A Clade C *pol* gene from isolate Du151 was chosen based on its 99% amino acid identity with the South African consensus sequence. This gene was modified at the active site of the reverse transcriptase encoding sequence to inhibit its activity, and the p51 fragment of this modified gene (SEQ ID NO:15) was engineered into a VEE

RNA replicon plasmid. The map of this *pol* plasmid is shown in Figure 4, and the nucleotide sequence of the plasmid is provided as SEQ ID NO:14. In Figure 6, expression of this POL p51 fragment (SEQ ID NO:16) in BHK cells is demonstrated (Western blot, lane 1), showing that the protein expressed in these cells is both the correct size and immunoreactive.

C. IMMUNOLOGICAL RESPONSE TO VRP-GAG VACCINE

Mice were injected subcutaneously in two doses, with 8-9 mice in each group. The mice were immunized once, then immunized a second time, with the same dose, 28 days later. Serum was collected the day prior to the first immunization, then at day 27 ("after 1st immunization) and at day 35 (after 2nd immunization).

The vigorous, antigen-specific humoral response of mice to the HIV-1 Clade C VRP-Gag vaccine described in Example 5.A.1. is presented in Table 1. Details of this assay are described in Example 7A.1.

TABLE 1. Humoral Response to VRP-Gag Vaccine

Dose:	Total Ab Titer (log_{10})
10^3 i.u. dose:	
after 1st immunization	1.3 +/- 0.1
after 2nd immunization	2.8 +/- 1.1
10^5 i.u. dose	
after 1st immunization	2.1 +/- 0.5
after 2nd immunization	4.1 +/- 0.6

A robust, Gag-specific response in mice was induced by the HIV-1 clade C VRP-Gag vaccine and is presented in Figure 9. Details of this assay are described in

Example 7A.3.

EXAMPLE 6

MANUFACTURING PROCESS FOR HIV VRP VACCINES

A. Manufacturing Process

Disclosed herein is a manufacturing process for VRP vaccines that is suitable for large-scale preparation of GMP-compliant (GMP = Good Manufacturing Practices) material for use in human clinical trials or for commercial manufacture. The process includes several steps and after each step (as appropriate), a set of "in process control" (IPC) assays or Release Tests (RT) is performed to confirm the successful completion of the step. The IPC/RT tests and process steps and the accompanying IPC assay(s) or RTs (described in more detail in Example 6D.1 and 6D.2) are as follows:

Process Step	**IPC/RT Tests and Process Steps**
Linearize 3 DNA plasmids	IPC: Check for linearity
In vitro RNA transcription	IPC: Size, integrity and concentration
Electroporation of certified Vero cell line	
Harvest culture fluids	IPC: Titration/Identity Test for replication-competent virus (RCV)
Pool the culture fluid	RT: Mycoplasma Adventitious virus PERT assay
Purification of bulk VRP by heparin affinity chromatography	IPCs: Heparin residual assay BSA assay Bovine IgG assay

Filtration of bulk VRP	RT: Test for RCV Titration/Identity Contaminating protein/DNA Sterility Endotoxin
Formulate, Fill, Release	RT: Titration/Identity Sterility General Safety

B. Preparation of plasmid DNAs

Stock solutions of replicon plasmid DNA, capsid helper plasmid DNA and glycoprotein helper plasmid DNA are produced in *Eschericia coli* XL2 Blue cells (Stratagene, cat# 200150). All plasmids harbor the kanamycin resistance gene marker. The three plasmid DNAs were manufactured and purified by PureSyn, Inc. (Malvern, PA) under appropriate GLP/GMP procedures, with a complete Batch Record with full traceability. Following fermentation and cell harvest, cell paste was lysed with base and plasmid DNAs were purified by ion pair chromatography on PolyFlo^{TM} separation media.

Prior to release by appropriate quality assurance/quality control oversight, each lot of each plasmid DNA is analyzed to confirm identity, purity and quality (Table 2). An approved certificate of analysis for each DNA is then established for each plasmid DNA lot.

Table 2. Plasmid DNA Release Tests

Test	Method	Specification
DNA homogeneity	Agarose gel electrophoresis	>90% supercoiled

E. coli genomic DNA	Southern Blot	< 50 μg/mg plasmid
E. coli RNA	Agarose gel electrophoresis	No detectable bands
Endotoxin	Limulus Amoebocyte Lysate (LAL)	< 0.1 EU/mg
Total protein	Abs 260/280	1.8-1.9
Sterility	Bioburden assay, USP23	< 1 CFU
Identity	Restriction enzyme analysis	Matches map

To produce HIV-VRP vaccine for clinical use, both replicon and helper plasmids are linearized by digestion at the unique Not I site and used as templates for synthesis of run-off transcripts. The quality of the transcription products (i.e., the replicon and the two helper RNAs) is evaluated by agarose gel electrophoresis.

C. Characterization of the Vero cells

Vero cells are used in the production of HIV-VRPs (WHO Vero MCB P139, BioReliance Inc., Rockville, MD). Vials contained approximately 1 x 10^7 cells/mL in a cryoprotectant solution of 90% fetal bovine serum and 10% dimethyl sulfoxide. A Cell Certification Summary is provided with each lot. BioReliance Inc. has filed a Master File with the FDA regarding the WHO Vero MCB P139.

Vials of WHO Vero MCB P139 cells are expanded into flasks. Each of the flasks is then expanded again in order to prepare the Master Cell Bank (MCB). The Working Cell Bank (WCB) is prepared from the MCB. The MCB is tested for purity and identity. The WCB is tested for adventitious agents (detection of mycoplasma and viruses). Viability tests are performed on both the MCB and the WCB.

Tumorigenicity tests are performed once at the end of the production period.

D. Electroporation

Vero cells are cotransfected by electroporation with RNA mixtures comprising replicon RNA transcripts encoding HIV-gag, VEE capsid helper RNA transcripts, and VEE glycoprotein helper RNA transcripts. The transfected cells are transferred to tissue culture vessels and incubated in well-defined culture medium. Following harvest, the HIV-Gag-VRP is purified from pooled culture fluid supernatants by affinity column chromatography. Prior to formulation and filling, purified, bulk HIV-Gag-VRP is tested for the presence of RCV.

E. Final formulated product

The HIV-Gag-VRP vaccine is vialed at four different doses. The material is filtered (0.22 μm) and added to vials at the appropriate concentration and volume, stoppered, quick-frozen and stored at –20° C.

F. Control tests of the Gag-VRP vaccine

1. In-Process Controls

Table 3 below summarizes the In-Process Controls performed during the manufacturing process of the HIV-Gag-VRP Vaccine.

Table 3. IPCs during the manufacture of HIV-Gag-VRP Vaccine

Test	Method	Target
Check for linearity	Agarose Gel electrophoresis	Report

Size, integrity and concentration of RNAs	Agarose Gel electrophoresis	Report
Titration/Identity	Indirect immunofluorescence assay(IFA), using standardized Gag-specific antibody preparation	Report
Test for RCV	CPE Assay	Report
Heparin Residual	Chromogenic Inhibitory Assay	Report
BSA residual	ELISA	Report
Bovine IgG Residual	ELISA	Report

2. Release tests

Tables 4 and 5 below summarize the release tests performed on the HIV-Gag-VRP Vaccine.

Table 4. Pool of the Culture Fluids

Test	**Method**	**Target**
Adventitious Virus (*in vivo*)	European guidelines	Negative
Adventitious Virus (*in vitro*)	5 cell lines	No growth
Mycoplasma	21CFR 610.30	No Growth
Reverse Transcriptase	PERT Assay	Negative

Table 5. Bulk VRP and Final Vial testing

Test	Method	Target Result
Replication competent virus (RCV)	Cytopathic effect (CPE) assay	Absence (in VERO cells, sensitivity is 1-10 pfu V3014)
VRP identity/ potency	Indirect immunofluorescence assay (IFA)	10^6 to 10^8 i.u. per mL
Cellular Protein Contaminant	Pierce BCA protein assay	Total protein content per dose
Cellular DNA Contaminant	Southern Blot or PCR	< 10 ng per dose
Sterility	21 CFR § 610.12	Pass
Endotoxin	LAL	< 5 EU/dose
General Safety	21 CFR § 610.11	Pass
Particulates	USP	Pass
Stability	IFA	10^6 to 10^8 i.u. per mL

EXAMPLE 7

PRECLINICAL STUDIES

Pilot lots are manufactured following written procedures (SOPs and STMs) and according to the manufacturing scheme described in Example 6. These pilot lots are prepared and used for two major tasks. The first one is a preclinical immunogenicity evaluation, which includes studies to assess the immune response and the cell-mediated

immune response in vaccinated animals. The second major task is a preclinical safety evaluation, which includes evaluations of system toxicity, hematopoietic and immune system toxicity, and local reactogenicity.

A. Immunogenicity Studies

A.1 Humoral Immune Response in Mice

Three groups of five female BALB/c mice (4-6 weeks of age) are inoculated subcutaneously with 10^5, 10^6, or 10^7 i.u. of the HIV-Gag-VRP at three time points: on day 0, and at weeks 4 and 8. The fourth group, Control Group, receives the vehicle only. Immediately prior to inoculation, and at weeks 3, 5, 8 and 10 post-inoculation, blood samples are collected for humoral immune response evaluations. Gag protein-specific serum antibody titers and seroconversion rates are measured by ELISA (Caley *et al.*, 1997) against purified, recombinant Gag protein. The source of the antigen is the homologous Clade C *gag* gene expressed in insect or mammalian cells. Antigen specificity also is confirmed by immunoblot analysis. Anti-VEE responses are monitored by ELISA (Johnston and Smith, 1988).

A.2 Humoral Immune Response in Rabbits

Three groups of five female New Zealand white rabbits are inoculated subcutaneously with 10^5, 10^6, or 10^7 i.u. of the HIV-Gag-VRP. The fourth group, Control Group, receives the vehicle only. Immediately prior to inoculation, and at weeks 3, 5,8 and 10 post-inoculation, blood samples are collected for humoral immune response evaluations.

Humoral immune responses are evaluated as described in Section A.1.

A.3 Cell-Mediated Immune Response in Mice

Three groups of five female BALB/c mice are inoculated subcutaneously with 10^5, 10^6, or 10^7 i.u. of the HIV-Gag-VRP at day 0 and day 28. The fourth group, Control Group, receives the vehicle only. Blood samples are collected at week 3 post-inoculation. Spleens are harvested for splenocyte collection on day 7 following the second inoculation for evaluation of cell-mediated immune responses.

The cell-mediated immune response is evaluated by determining the ability of splenic T cells from immunized mice to proliferate *ex vivo* in the presence of either Gag protein or Gag peptide(s). The ability of splenic T and CD4+ T cells to produce interferon-γ and interleukin-4 respectively, is determined. Finally, the ability of cytotoxic T lymphocytes to lyse target cells that present murine major histocompatibility complex class-I restricted epitopes for HIV-1 Clade C Gag protein is measured (see Betts *et al.*, 1997 for methods)

B. Safety Study

Three groups of six male and six female New Zealand white rabbits are inoculated subcutaneously with 10^4, 10^6, or 3 x 10^7 i.u. of the HIV-Gag-VRP. The fourth group, Control Group, receives the vehicle only. Animals receive four injections at week 0, week 3, week 6 and Week 9. Half of the animals are sacrificed two days after the last injection (week 9) and the other half at three weeks after the last injection (week 12). Similar studies are performed in mice with a high dose at 10^8 i.u. This level is 10-100 times the likely primate dose, based on efficacy studies in rhesus macaques.

In addition to system toxicity (record of mortality/morbidity, body temperature, body weight, food consumption and ophthalmic examinations), hematopoietic toxicity is evaluated by quantitating cellular components of peripheral blood, and immune system toxicity is assessed by histopathologic evaluation of the lymphoid organs. Local reactogenicity is evaluated by examining the injection sites grossly and

microscopically to determine irritation potential. Serum samples are also tested for the presence of replication competent virus by blind passage in cell culture.

C. In *Situ* Hybridization Study in Mice

Three groups of five female BALB/c mice are inoculated subcutaneously with 10^5, 10^6, or 10^7 i.u. of the HIV-Gag-VRP. The fourth group, Control Group, receives the vehicle only. A single injection is performed in each group.

To verify expression of HIV-GAG-VRP in lymphoid tissue, the draining lymph nodes, spleen, and thymus of the mice are examined by *in situ* hybridization at 24 hours and 48 hours after the single inoculation.

EXAMPLE 8

Heparin Affinity Chromatography of VRPs

Generally, the majority of contaminating protein is non-VEE protein from the conditioned media. Heparin column capacity requirements for GMP manufacturing runs are therefore based on the volume of conditioned media, rather than the concentration of VRPs. Column parameters are optimized at room temperature, but variations in temperature do not greatly affect performance. The expected yields of VRPs can range from 50% to > 90%.

While only minimal leaching of heparin from the columns has been detected, GMP requirements stipulate that a residual heparin assay be performed as an IPC test following the chromatography step.

A. Pharmacia HiTrap® Heparin

Five mL columns of Pharmacia HiTrap® Heparin (cat no. 17-0407-01,

Amersham Pharmacia Biotech), pre-equilibrated with 25 mM HEPES/0.25 M NaCl, pH 7.5, were loaded with HIV-Gag-VRPs produced in Vero cells. After column washing with the equilibration buffer, VRPs were eluted with a 15 column volume gradient from 0.25 – 1.0 M NaCl gradient in 25 mM HEPES, pH 7.5. The HIV-Gag-VRPs eluted at a conductivity of approximately 48 mS/cm. The wash step was optimized (based on the A_{280} peak) at a NaCl concentration between 0.25 M and 0.3 M.

B. Heparin Sepharose 6 Fast Flow® resin

Heparin Sepharose 6 Fast Flow® resin (catalog no. 90-1000-2; Amersham Pharmacia Biotech) is supplied as a bulk resin which allows various size columns to be packed as needed. Fast Flow® resins have the advantages of excellent flow characteristics and ability to be sanitized with sodium hydroxide solutions, which are particularly useful in a GMP manufacturing process. A 6 mL column was prepared by packing the Heparin Sepharose 6 Fast Flow® bulk resin in a BioRad® Econo-Column chromatography column, which was then pre-equilibrated with 25 mM HEPES/0.12 M NaCl, pH 7.5. VRPs were loaded onto the column, which was then washed with the equilibration buffer. Initial experiments indicated that the VRPs eluted at a lower conductivity (36 mS/cm) with this resin as compared to the HiTrap® Heparin, so the wash conditions were modified accordingly. The VRPs were eluted from the Fast Flow® resin with a 15 column volume gradient from 0.12 M to 1 M NaCl in 25 mM HEPES, pH 7.5.

EXAMPLE 9

Virosome Formation

The feasibility of virosome formation is demonstrated in a series of experiments in which replicon RNA and RNA encoding the glycoprotein E1 and E2 genes (glycoprotein helper) were first transfected into BHK cells by electroporation. After 18-24 hours, cell supernatants were harvested and tested for the presence of virosomes as described briefly below.

Cell Culture

BHK cells were used as a cell substrate and were maintained in growth medium (alpha-MEM (Life Technologies), supplemented with 10% Fetal Bovine Serum (HyClone), 1x Glutamine (Life-Technologies)), in an atmosphere of 5% CO_2 at 37°C. Prior to electroporation, cells were detached from the cell culture vessel using 0.05% trypsin-0.53 mM EDTA solution (Life Technologies). Trypsin was neutralized with growth medium, and cells were washed twice with cold Phosphate-Buffered Saline (PBS, BioWhittaker) and resuspended at a concentration of 1.5 x 10^7 cells/ml.

RNA Transcription, Electroporation and Virosome Harvest

Plasmid DNA pVR-GFP (green fluorescent protein) was linearized using restriction endonuclease NotI (New England Biolabs) as recommended by the manufacturer. DNA was extracted with phenol:chloroform:iso-amyl alcohol (25:24:1, Gibco BRL) and precipitated with ethanol, following the addition of NH_4Ac to 2.5 M final concentration. RNA was synthesized in an *in vitro* transcription reaction using an mMessage mMachine® kit (Ambion) as recommended by the manufacturer. This RNA, without further purification, was used to transfect BHK cells. Helper RNA was prepared in a similar fashion. A BHK cell suspension in PBS (0.8 mL, 1.2 x 10^7 cells) was mixed with 10 μg of each RNA, and the mixture was electroporated. Electroporation settings for Gene-Pulser® (Bio-Rad Laboratories) were: 850 V, 25 μF, 3 pulses. Culture supernatant was collected at 18-24 hr post-electroporation and clarified by centrifugation for 10 min at 1000 rpm.

Titration of Virosomes

The presence of infectious virosome particles was demonstrated using an immunofluorescence assay to titer the virosomes by detecting the fluorescence of the GFP encoded by the replicon RNA in the virosomes. Serial dilutions of the cell culture supernatant were added to 12-well plates of BHK cells. Following an 18-24 hour

incubation in an atmosphere of 5% CO_2 at 37°C, the medium was removed from each plate. Virosome infectious titer was then determined by counting the number of green-fluorescent single cells at a particular dilution, followed by a back-calculation to determine total infectious units (i.u.) per mL. A final titer of 440 i.u./mL was collected.

Confirmation of virosome identity

Three independent experimental methods were used to determine that the infectious particles were in fact virosomes, rather than replication competent viral particles or naked RNA being carried over from the electroporated cells.

i) The virosome-containing supernatant was passaged a second time by removing the cell supernatant from the 12-well plate used for titration and placing this supernatant onto a fresh monolayer of BHK cells. At 18-24 hours post-passage, the monolayer was examined under U/V fluorescence and found to contain 0 (zero) GFP-positive cells, indicating the infectious particles produced using this method can undergo only a single round of replication, a critical characteristic of a virosome.

ii) To establish that the infectious titer detected following virosome packaging was not due to carry-over of RNA used in the electroporation, the supernatant was treated with RNase A (Invitrogen) at a concentration of 100 μg/mL for 15 minutes at 37°C. The treated and untreated control supernatants were titered according to the methods outlined above. The RNase-treated sample contained 400 i.u./mL and the control group had 440 i.u./mL, indicating that the RNAse treatment had no significant effect on virosome titer.

iii) To establish that the infectious particles were enveloped in the E1 and E2 glycoproteins, anti-VEE mouse serum was used to treat the cell supernatant in a neutralization assay. As a control, normal mouse serum was used to treat the virosome supernatant. In addition, VEE replicon particles expressing GFP were used in the assay, the infectivity of which is known to be inhibited by this serum.

	Particle Titer (i.u./mL)		
	Anti-VEE serum	Normal Mouse Serum	No serum
Virosome Supernatant	20	440	530
VRP-GFP	0	530	890

The infectivity of the virosomes was inhibited similar to that of VRP-GFP, indicating that the virosome particles were enveloped by the E1 and E2 glycoproteins.

These examples clearly demonstrate the ability to produce infectious virosome particles comprising replicon RNA enveloped with only the alphavirus E1 and E2 glycoproteins. Testing confirmed that these virosomes are infectious agents, but that they undergo only a single round of replication, as indicated by the inability to passage the agent. In addition, the agents contained the E1 and E2 glycoproteins, as evidenced by the ability to block infection with only VEE specific serum. Finally, the infectious RNA is protected from RNase enzymatic digestion, indicating an enveloped particle.

The natural lipid content in BHK cells is primarily non-cationic. Virosomes made in a completely cell free system can be made by using one or more non-cationic lipids, such as lecithin (phosphatidycholine).

EXAMPLE 10
PHASE I CLINICAL PROTOCOL

Phase I Safety and Immunogenicity Trial of an HIV Subtype C Gag-VEE Replicon Particle Vaccine in HIV-1 Seronegative Human Subjects

A Phase I trial is conducted to evaluate the safety and immunogenicity of the HIV Gag-VRP prototype vaccine component in healthy seronegative adult volunteers. The doses are selected based on preclinical studies in rodents and nonhuman primates. The schedule mimics previous preclinical efficacy studies with the SIV model that

demonstrated the capacity of SIV-VRP to induce SIV specific neutralizing antibodies and CTL.

Purpose: To evaluate the candidate vaccine component in an open-labeled, placebo-controlled study.

Subjects: Healthy adult volunteers without a history of identifiable high-risk behavior for HIV-1 infection as determined by a comprehensive screening questionnaire.

No. Subjects: **40**

Route: Subcutaneous injection

Scheme: The volunteers are arranged in four groups, ten subjects per group. In each group, two subjects receive a placebo, while the other eight subjects receive either 10^4, 10^6, 10^7, or 10^8 i.u. of HIV-Gag-VRPs. Subjects are vaccinated on day 0, day 30, and day 120.

Estimated Duration: Forty weeks

A. SELECTION of SUBJECTS

Subjects are healthy HIV-1 seronegative adults who fully comprehend the purpose and details of the study as described in the informed consent. Subjects whom either themselves or whose sexual partners have identifiable higher risk behavior for HIV-1 infection are not eligible. Higher risk behavior is determined by a prescreen series of questions designed to identify risk factors for HIV-1 infection. An assessment of absolute exclusion criteria using the self-administered and interview questions is conducted. Subsequently, investigators proceed with phlebotomy, history and physical examination, and final questions regarding sexual behavior and other practices. Eligibility determinations for the trial depend on results of laboratory tests and answers to these self-administered and interview questions.

The criteria used to define low risk behavior are as follows:

EITHER ALL OF THE FOLLOWING:

1. No newly acquired higher risk associated STD in the last six months
2. No possibly safe or unsafe sex with a known HIV+ individual or an active injection drug user in the past six months
3. No unsafe sexual activity
4. Possibly safe sexual activity with two or fewer partners within the last six months
5. No injection drug use

OR BOTH OF THE FOLLOWING:

1. Mutually monogamous relationship with a known or presumed HIV seronegative partner for the last six months
2. No injection drug use

A.1 Inclusion Criteria

Age: 18-60

Sex: Male or Female *[For females, negative pregnancy test at time of entry and assurance that adequate birth control measures will be used for one month prior to immunization and the duration of the study]*

Normal history and physical examination

Lower risk sexual behavior as defined above.

Normal complete blood count and differential defined as:

- Hematocrit 34% for women; 38% for men
- White count 3500 cells/mm^3 with normal differential
- Total lymphocyte count 800 cells/mm^3
- Absolute CD4 count 400 cells/mm^3
- Platelets (150,000-550,000)

Normal ALT (~ 1.5 x institutional upper normal limit) and creatinine

(1.6 mg/dl)

Normal urine dipstick with esterase and nitrite

Negative for hepatitis B surface antigen

Negative ELISA for HIV within eight weeks of immunization

Availability for follow-up for planned duration of the study (68 weeks)

A viable EBV transformed autologous B cell line

A.2 Exclusion Criteria

History of immunodeficiency, chronic illness, malignancy, autoimmune disease, or use of immunosuppressive medications

Medical or psychiatric condition or occupational responsibilities which preclude subject compliance with the protocol

Subjects with identifiable higher risk behavior for HIV infection as determined by screening questionnaire designed to identify risk factors for HIV infection; specific exclusions include:

History of injection drug use within the last 12 months prior to enrollment.

Higher risk sexual behavior defined as one or more of the following behaviors:

1. A newly acquired higher risk associated STD within the past six months
2. Possibly safe or unsafe sex with a known HIV+ individual in the past six months
3. Possibly safe sexual activity with twelve or more partners in the past six months
4. Unsafe sexual activity with four or more partners within the past six months.

Live attenuated vaccines within 60 days of study [NOTE: Medically indicated subunit or killed vaccines (e.g., influenza, pneumococcal) are not exclusionary, but should be given at least two weeks away from test article immunizations.]

Use of experimental agents within 30 days prior to study

Receipt of blood products or immunoglobulin in the past six months

Active syphilis [NOTE: If the serology is documented to be a false positive or due to a remote (>six months) treated infection, the volunteer is eligible]

Active tuberculosis [NOTE: Volunteers with a positive PPD and a normal chest X-ray showing no evidence of TB and not requiring INH therapy are eligible.]

History of anaphylaxis or other serious adverse reactions to vaccines

Prior receipt of HIV vaccines or a placebo recipient in an HIV vaccine trial

Pregnant or lactating women

B. SAFETY and IMMUNOGENICITY MONITORING

Safety is evaluated by monitoring volunteers for adverse reactions during the course of the trial. Volunteers are followed for a total of 26 weeks post-final inoculation. The main toxicity associated with the subcutaneous injection in this study is that associated with subcutaneous injection of any immunogen, i.e., pain, redness and swelling at the injection site, as well as the possibility of fever, chills, aches and pains and perhaps fatigue.

Safety monitoring includes periodic review of data from the trial with particular emphasis on monitoring for adverse reactions including the following evaluations:

Hematologic: CBC, differential, platelets

Hepatic/renal: ALT, creatinine, urinalysis

Neurologic: headache, paralysis, anxiety, confusion, weakness, tremors.

Systemic symptoms: fever, gastrointestinal complaints, myalgia, malaise, fatigue, headache, anaphylaxis, immune complex disease, and other hypersensitivity reactions

Local toxicity at the site of injection: e.g., pain, tenderness, erythema, regional lymphadenopathy, limitation of limb movement

The immunogenicity monitoring includes the following immunological assays,

all utilizing HIV Subtype C based reagents:

Humoral responses:

HIV Subtype C Gag-specific ELISA

Anti-VEE ELISA

Cellular immune responses:

Standard cell-killing assay (i.e., chromium release) to measure CD8+ Gag-specific CTL activity

ELISPOT assay to measure IFN-?

Mucosal immune responses:

Standardized assay for assessment of Gag-specific IgA

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

REFERENCES

Barany F. 1985. Single-stranded hexameric linkers: a system for in-phase insertion mutagenesis and protein engineering. *Gene* **37**(1-3):111-23.

Betts, M. R., J. Krowka, C. Santamaria, K. Balsamo, F. Gao, G. Mulundu, C. Luo, N. N'Gandu, H. Sheppard, B. H. Hahn, S. Allen and J. A. Frelinger. 1997. Cross-clade human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte responses in HIV-infected Zambians. *J. Virol.* **71**:8908-8911.

Caley, I. J., M.R. Betts, D.M. Irlbeck, N. L. Davis, R. Swanstrom, J.A. Frelinger and R. E. Johnston. 1997. Humoral, mucosal and cellular immunity in response to an HIV-

1 vaccine candidate. *J. Virol.* **71**:3031-3038.

Davis *et al.* 1980. In: Microbiology, 3d ed., p. 132.

Davis, N.L., L.V. Willis, J.F. Smith and R.E. Johnston. 1989. *In vitro* synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: Analysis of a viable deletion mutant. *Virology* **171**:189-204.

Davis, N. L., L. V. Willis, J. F. Smith, G. Greenwald and R. E. Johnston. 1990. *In vitro* synthesis of infectious VEE virus RNA from a cDNA clone: Analysis of a viable deletion mutant and mutations affecting virulence. In: Vaccines 90, Cold Spring Harbor Press, Cold Spring Harbor, NY. pp. 109-113.

Davis, N. L., N. Powell, G. F. Greenwald, L. V. Willis, B. J. Johnson, J. F. Smith and R. E. Johnston. 1991. Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: Construction of single and multiple mutants in a full-length cDNA clone. *Virology* **183**:20-31.

Davis, N. L., K. W. Brown, G. F. Greenwald, A. J. Zajac, V. L. Zacny, J. F. Smith and R. E. Johnston. 1995. Attenuated mutants of Venezuelan equine encephalitis virus containing lethal mutations in the PE2 cleavage signal combined with a second-site suppressor mutation in E1. *Virology* **212**:102-110.

Davis, N. L., K. W. Brown and R. E. Johnston. 1996a. A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. *J. Virol.* **70**:3781-3787.

Davis, N. L., P. Pushko, K. W. Brown, P. C. Charles, I. J. Caley, M. Parker, G. Ludwig, J. F. Smith and R. E. Johnston. 1996b. Immunization against influenza with attenuated Venezuelan equine encephalitis virus vectors. In: Options for the Control of Influenza, III, L. E. Brown and A. W. Hampson, eds. Elsevier, Amsterdam. pp.803-

809.

Davis, N. L., I. J. Caley , K. W. Brown, M.R. Betts, D.L. Irlbeck, K.M. McGrath, M.J. Connell, D.C. Montefiori, J.A. Frelinger, R. Swanstrom, P.R. Johnson and R. E. Johnston. 2000. Vaccination of macaques against pathogenic simian immunodeficiency virus with Venezuelan equine encephalitis virus replicon particles. *J. Virol.* **74**:371-378

Grieder, F. B., N. L. Davis, J. F. Aronson, P. C. Charles, D. C. Sellon, K. Suzuki and R. E. Johnston. 1995. Specific restrictions in the progression of Venezuelan equine encephalitis virus induced disease resulting from single amino acid changes in the glycoproteins. *Virology* **206**:994-1006.

Hevey, M., D. Negley, P. Pushko, J. Smith and A. Schmaljohn. 1998. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. *Virology* **251**:28-37.

Hirsch, V., T.R. Fuerst, G. Sutter, M.W. Carroll, L. C. Yang, S. Goldstein *et al.* 1996. Patterns of viral replication correlate with outcome in SIV-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. *J. Virol.* **70**:3741-3752.

Johnston, Robert E. and Jonathan F. Smith. 1988. Selection for accelerated penetration in cell culture co-selects for attenuated mutants of Venezuelan equine encephalitis virus. *Virology* **162**:437-443.

Johnston, R. E. and C. J. Peters. 1996. *Alphaviruses*. In: Virology, Third Edition, B. N. Fields, D. M. Knipe and P. M. Howley, eds., Raven Press, New York. pp. 843-898.

Kinney, R.M., B.J.B. Johnson, J.B. Welch, K.R. Tsuchiya and D.W. Trent. 1989. The full-length nucleotide sequences of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and its attenuated vaccine derivative, strain TC-83. *Virology*

170:19-30.

Kinney, R.M., G-J. Chang, K. R. Tsuchiya, J. M. Sneider, J. T. Roehrig, T. M. Woodward and D. W. Trent. 1993. Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5'-noncoding region and the E2 envelope glycoprotein. *J. Virol.* **67**:1269-1277.

Kunkel. 1985. *Proc. Natl. Acad. Sci.* USA 82:488.

Paredes, A.M., D.T. Brown, R. Rothnagel, W. Chiu, R.J. Schoepp, R.E. Johnston and B.V.Prasad. 1993. Three-dimensional structure of a membrane-containing virus. *Proc. Natl. Acad. Sci.*, USA **90**:9095-9099.

Pushko, P., M. Parker, G. V. Ludwig, N. L. Davis, R. E. Johnston and J. F. Smith. 1997. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes *in vitro* and immunization against heterologous pathogens *in vivo*. *Virology* **239**:389-401.

Rosenberg, A.H., *et al.* 1987. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene*. **56**(1): p. 125-35.

Schlesinger, S. and M.J. Schlesinger. 1990. *Replication of Togaviridae and Flaviviridae*. In: Virology, Fields, B.N. and Knipe, D.M. (eds.) Raven Press. pp. 697-711.

Strauss *et al.* 1990. *Seminars in Virology* 1:347.

Strauss, J.H. and E.G. Strauss. 1994. The alphaviruses: Gene expression, replication, and evolution. *Micro. Rev.* **58**:491-562.

Studier, F.W., *et al.* 1990. Use of T7 RNA polymerase to direct expression of cloned

genes. 1990. *Methods Enzymol.* **185**:60-89.